Artificial Nucleases

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The oxidation of DNA and RNA provides a facile approach for investigating the interaction of nucleic acids with proteins and oligonucleotides. In this article, we have outlined our understanding of the mechanism of DNA scission by 1,10-phenanthroline – copper(I) in the presence of hydrogen peroxide. We also discuss results obtained by using 1,10-phenanthroline – oligonucleotide conjugates in probing the size of the transcriptionally active open complex. Finally, we outline an effective method for converting DNA-binding proteins into site-specific modification agents by using 1,10-phenanthroline – copper(I).

KEYWORDS:

copper · DNA cleavage · DNA recognition · nucleic acids · oligonucleotides

1. Introduction

The biological world may have begun with reactive RNAs, but the long-term stability of the genetic message was installed in the hydrolytically stabile DNA. However, the lability of DNA to oxidation has frequently been overlooked and reagents in which redox-active coordination complexes are tethered to both proteins and oligonucleotides have not received much attention. We have discovered one type of these reagents, and we feel that these reagents may play an important role in telling us about genomic structure.[1]

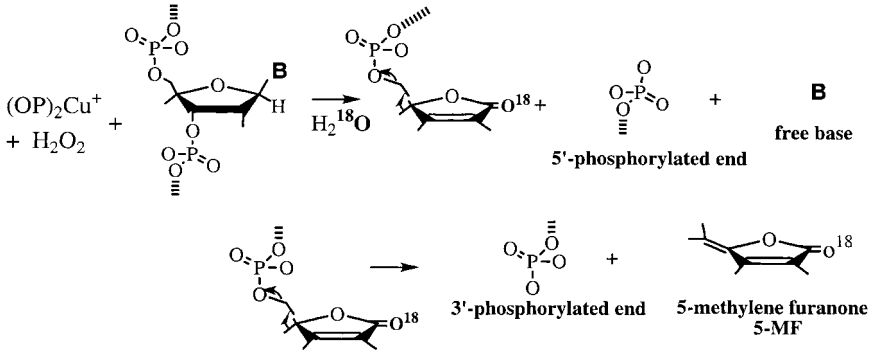

Figure 1. *Mechanism of scission of DNA by 1,10-phenanthroline – copper(I) and hydrogen peroxide. (Reprinted with permission from* J. Am. Chem. Soc. ***1997,*** 119, *1135 – 1136. Copyright 1997 American Chemical Society.)*

2. Cleavage of DNA

Bis(1,10-phenanthroline) cuprous chelate ($[(OP)_2Cu]^+$; OP = 1,10-phenanthroline, *ortho*-phenanthroline) cleaves DNA by one predominant mechanism in which the initial site of attack involves oxidative attack at atom C1′ of the deoxyribose.[2, 3] The untargeted reaction involving 1,10-phenanthroline or derivatives having substituents at positions other than the 2- and 9-positions, and copper(I) ions, rapidly degrades DNA in the presence of trace levels of hydrogen peroxide and 3-mercaptopropionic acid. This reaction was discovered accidentally during the course of experiments actually designed to find a phenanthroline derivative that inhibited *Escherichia coli* DNA polymerase I by coordination to an active-site zinc ion.[4–7] Subsequent work determined that zinc ion was not present and the reason for the effective inhibition was the destruction of poly(dA-dT) to generate phosphorylated products of the DNA that were binding to the active site of the enzyme, thus blocking polymerization.[8] The reactivity of DNA with oxidizing agents such as the 2:1 1,10-phenanthroline – copper(I) complex and hydrogen peroxide ($[(OP)_2Cu]^+ + H_2O_2$) is hypothesized in Figure 1. Detailed analyses of the products indicate that the C1′ group of the deoxyribose located in the minor groove is likely the initial site of attack because carrying the reaction out in $H_2{}^{18}O$ leads to the incorporation of ^{18}O in the carbonyl group of 5-methylene furanone.[3] Other possibilities may be envisioned, but this appears to be the most likely pathway.

Important properties of the DNA scission reaction include a 3′ stagger of the two strands and the absolute dependence of the reaction on hydrogen peroxide when a cleavage-competent 1,10-phenanthroline is used as the scission reagent. In the absence of targeting, the reaction preferentially cleaves the B-form of DNA. Less effectively, the reagent cleaves A-DNA and the chelate is unreactive to Z- and single-stranded (ss) DNA.[9] Untargeted $[(OP)_2Cu]^+$ does not cleave DNA at all sequence

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positions at the same rate. Analysis of the intensity of scission of all nucleotides has indicated that the decreasing order of reactivity is G > A > T > C. The 5′ neighbor of the cleaved DNA has a very substantial influence on the rate of cleavage; the neighboring nucleotide 3′ to the cleavage site has a very minor effect.[10, 11]

Given the importance of double-stranded DNA to the cleavage reaction, we assumed that double-stranded RNA might be an excellent substrate for the scission reaction. This proved exactly contrary to what we observed. RNA is cleaved only in single-stranded regions or close to single-stranded loops.[12] The chemistry of RNA scission has not been extensively developed because it is unclear if the rates of scission can be attributed to the extra 2′-OH group on the ribose or to other factors. Possibly, the preliminary noncovalent form of the $[(OP)_2Cu]^+$ complex is not exceptionally reactive because of an unproductive geometry.[12, 13]

3. Targeting transcription complexes

3.1. Reactivity of 5-phenyl-1,10-phenanthroline and 1,10-phenanthroline

The chemical nuclease activity has two distinct reactivities toward active transcription complexes. First, we demonstrate that the copper coordination complexes of 1,10-phenanthroline and 5-phenyl-1,10-phenanthroline are capable of detecting induced changes in DNA caused by the binding of RNA polymerase. Second, we show that 1,10-phenanthroline linked to the termini and interior of RNAs is competent to cleave the ssDNA created by the binding of RNA polymerase.

Both 5-phenyl-1,10-phenanthroline and 1,10-phenanthroline themselves, without modification, attack activated promoters in addition to serving as effective footprinting reagents. The unusual value of the reagents is that they provide an ideal means for observing functionally important conformational changes during transcription. For example, the addition of $[(OP)_2Cu]^+$ complex in the absence of Mg^{2+} demonstrates that the active RNA polymerase enzyme binds to the *lac* promoter, but does not activate transcription. This is true for both prokaryotic and eukaryotic transcription units.[14, 15] When Mg^{2+} is added to the *lac* UV5 promoter, new bands representing the cut sites at positions −6, −5, −4, and −3 are observed in the polyacrylamide gel electrophoresis (PAGE) pattern. These bands appear asymmetrically and are only observed for the template strand. The series of cut sites does not stop at this point. With 5-phenyl-1,10-phenanthroline and copper(I) ions in the presence of a thiol, ApA and UTP cause the cleavage sites to progress to the −4 to −2 region, whereas ATP and GTP induce the sites of scission to progress to the +2 to +4 region. Upon addition of a 9-mer RNA with the chain-terminating 3′-OMe cytosine, a doublet peak of DNA scission appears at positions +9 and +10.[16] The reasons for scission must be attributable to the complementary dependence of the DNA–RNA polymerase interface and the flexibility of the coordination complex in the presence of RNA.

3.2. Oligonucleotide-directed enzyme inhibitors

The surprising observation of the affinity of an oligonucleotide for the open complex raised provocative questions with regard to transcription initiation. Most significant is the possibility of designing oligonucleotides complementary to open complexes and using them as *hybridizable gene-specific inhibitors*.[17] Our initial goal was to determine if the complementary oligonucleotides had any affinity at all for the open complex. Is it possible to make complementary oligonucleotides that bind to the top or bottom strand? Can the oligonucleotides transport themselves into cells at sufficient concentrations to be effective inhibitors?[18, 19]

We addressed these questions initially with the *lac* UV5 promoter. By chance, we synthesized the oligonucleotide 5′-UGGAA-3′. This oligonucleotide was prepared chemically with authentic RNA monomers. According to the sequence of the *lac* UV5 promoter, this ribooligonucleotide should be complementary to 3′-ACCTT-5′, the template strand of *lac* UV5 from the −3 to +2 positions. In the presence of RNA polymerase, we found that it bound to the template with high affinity and inhibited polymerization. In addition, in the presence of copper(I) and ascorbic acid, 1,10-phenanthroline–UGGAA cleanly cleaved the target DNA within the transcription bubble. The general picture that we show here (Figure 2) is illustrated with 5′-GUGGA-3′ that is as effective an inhibitor as 5′-UGGAA-3′.

Although we were pleased that inhibition of transcription could be achieved with a 5-mer, we made an intensive effort to synthesize abiological oligonucleotides as inhibitors of both the *lac* UV5 and *trp* EDCBA templates. This also provided an extremely useful test for specificity. The oligonucleotide for *lac* UV5 was 5′-UGGAA-3′ and that for *trp* EDCBA was 5′-CGCAA-3′. In each case, the oligonucleotides were synthesized so that they had 2′-methoxyribose nucleotides and 2′-deoxy termini.[18] With these unusual termini, there was no possibility of enzymatic extension of the oligonucleotides by RNA polymerase. We found that for the promoter targeted by its complementary oligonu-

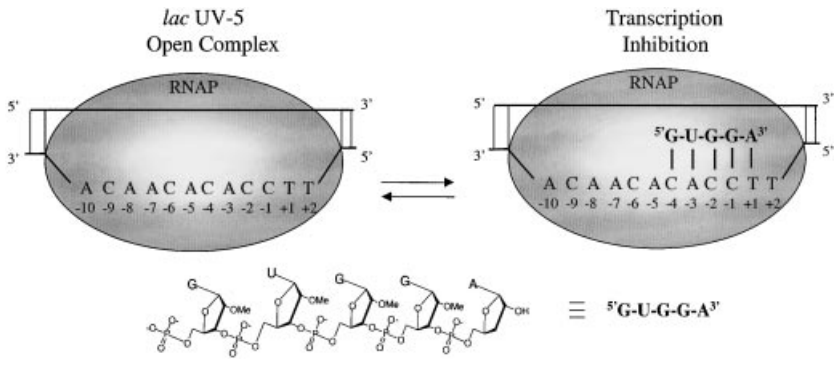


Figure 2. *Targeting the open complex with an RNA oligonucleotide linked to 1,10-phenanthroline–copper(I). RNAP = RNA polymerase.*

cleotide (e.g. *lac* UV5 with 5′-UGGAA-3′), inhibition of greater than 85% of the targeted transcription was observed. The inhibition of the noncognate promoter was only 10 – 15% under comparable conditions. The results were symmetric, as comparable results were observed in targeting the *trp* EDCBA promoter (Figure 3). It is important to recognize that with these

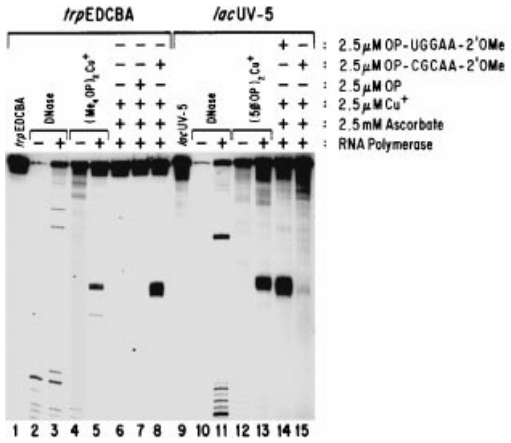


Figure 3. *Scission of* trp *EDCBA and* lac *UV5 by pentamers in the presence of RNA polymerase. Lane 1:* trp *EDCBA promoter fragment labeled on template strand; lanes 2 and 3: DNase I footprint of the promoter and open complex, respectively; lanes 4 and 5: 3,4,7,8-tetramethyl-1,10-phenanthroline – copper(I) ([(Me$_4$OP$_2$)Cu$^+$]) footprint of the* trp *EDCBA promoter and open complex, respectively; lane 6: copper(I) control (2.5 μM); lane 7: 1,10-phenanthroline – copper(I) control (2.5 μM – 2 μM); lane 8: site-specific scission directed by 2.5 μM 1,10-phenanthroline – r(CGCAA)-(2′OMe)$_{2-5}$ to* trp *EDCBA; lane 9:* lac *UV5 promoter labeled on the template strand; lanes 10 and 11: DNase footprinting of the* lac *UV5 promoter and open complex, respectively; lanes 12 and 13: 5-phenyl-1,10-phenanthroline – copper(I) footprinting of the* lac *UV5 promoter and open complex, respectively; lane 14: site-specific scission directed by 2.5 μM 1,10-phenanthroline – r(UGGAA)-(2′OMe)$_{2-5}$ to the* lac *UV5 promoter (positive control); lane 15: site-specific scission directed by 2.5 μM 1,10-phenanthroline – r(CGCAA)-(2′OMe)$_{2-5}$ to the* lac *UV5 promoter (negative control). Scission in lanes 6 – 8, 14, and 15 was initiated by addition of 2.5 μM sodium ascorbate and continued for 25 min at 37 °C. (Reprinted with permission from* J. Am. Chem. Soc. ***1997****,* 119, *5746 – 5747. Copyright 1997 American Chemical Society.)*

two oligonucleotides there are differences at only two of the five nucleotides in the pentamer. Only the 1 and 3 positions of the sequence are different. These simple changes indicate that the specificity of the oligonucleotides is very stringent.

4. Length dependence of inhibition

The extension of this work requires further investigation of the dependency of the binding on the structure of the oligonucleotide inhibitor. One key question is the length of the oligonucleotide that can hybridize to the open complex of *lac* UV5. To address this, a series of oligonucleotide inhibitors was prepared that ranged from the 5-mer to an 11-mer. All oligonucleotides contained the following substitutions: 3′-deoxy-2′-hydroxyadenosine, a 5′-phosphate and 2′-methoxyribose moieties throughout. In this series of experiments, we also prepared an identical nucleotide, 5′-UCCAC-3′. This oligonucleotide has the same modifications as above and is complementary to the nontemplate DNA strand.

Our results were dramatic. The 5- and 6-mers remained inhibitory for both promoters. With their respective promoters, they showed high affinity for the open complex. Longer oligonucleotides failed to bind with any affinity to the RNA polymerase – *trp* EDCBA or –*lac* UV5 promoter complexes. These longer dead-end oligomers did not bind to the DNA strand although they contained the inhibitory 5-mer sequence. Somewhat surprisingly 5′-UCCAC-3′, which has the opposite polarity of 5′-UGGAA-3′, had no inhibitory effect when measured either

5′-UGGAA-3′
3′-GACCU-5′

directly by kinetic methods or by indirect scission using 1,10-phenanthroline – 5′-UGGAA-3′ to probe the transcription complex for 5′-UCCAC-3′ binding. Our failure to observe inhibition indicates that the top strand of DNA inside the transcription bubble is not accessible to oligonucleotide inhibitors (Figure 4).

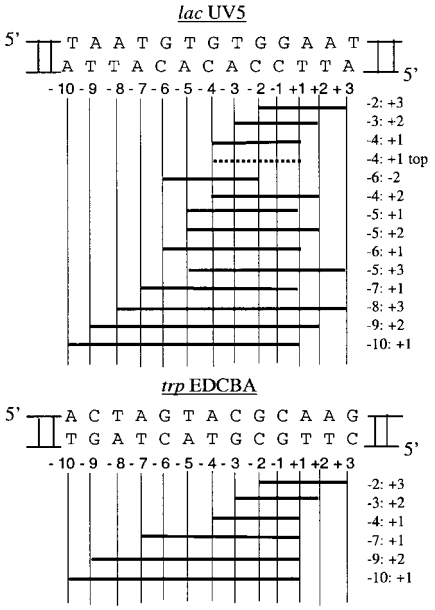


Figure 4. *Size dependence of transcription inhibitors. The black bars represent oligoribonucleotide analogues with sequences complementary to the template strand of either the* lac *UV5 or* trp *EDCBA promoters. In each case the oligonucleotide analogues have been synthesized with 3′-deoxy-2′-hydroxyadenosine, a 5′-phosphate, and 2′-methoxyribose moieties. One strand (dashed line) is complementary to the RNA (upper) strand from −4 to +1. This data was obtained by the indirect cleavage method in which scission was carried out with 5′-1,10-phenanthroline – UGGAA-3′ (all interior nucleotides possess 2′-methoxyribose). (Reprinted with permission from* Proc. Natl. Acad. Sci USA ***2000****,* 97, *3136 – 3141. Copyright 2000 National Academy of Sciences, U.S.A.)*

5. Conformation of the inhibited complex

What is the structure of the inhibited complex formed in the presence of RNA polymerase – *lac* UV5 and an inhibitory pentamer? To address this question, we prepared three oligonu-

cleotides in which the OP moiety was linked either to the 5′ end of 5′-GUGGA-3′, the first internal uridine, or the 3′ end (Figure 5 A). Scission was then carried out. The results are shown in Figure 5 B. As the 1,10-phenanthroline moiety is moved, there is a corresponding shift in the sites of scission as one proceeds from the 3′ end to the 5′ end. The most informative set of cleavage results is the one in which the U was labeled with OP at the 2′-position of the ribose. In this series of experiments, this substitution showed a 2 – 3 base pair 3′ stagger.

Our results strongly indicate that the inhibited complex forms an antiparallel helical structure. However, the lengths of the oligonucleotides are not sufficient to resolve the A-form structure from the B-form structure. Given the predilection of RNAs to form A-form structures both in heteroduplexes and in RNA helices, the results strongly suggest that the inhibitory complex must be closely related to the A-form of the helix.

6. Inhibition by phosphorothioates

The restriction in the length of oligonucleotides that can enter the open complex and the specificity of binding to the open complex sets strong constraints in the design of tight-binding inhibitors for the *lac* UV5 promoter and presumably other promoters. Oligonucleotides that do not interact with the open complex include the corresponding peptide nucleic acid of 5′-GUGGA-3′, morpholino derivatives of the same pentamer, the N^3-P^5 phosphoramidate, and the DNA of the identical sequence. In addition, various modifications of the 5′-phosphate have some effects on K_i, but do not change the free energy of binding significantly. The most dramatic effect that we have noticed is the increased inhibition by the phosphorothioate backbone containing 2′-methoxyribose substituents in the 5′-GUGGC-3′ pentamer. The IC_{50} value of this inhibitor approaches 3 μM, strongly suggesting that this modification may be the direction that we want to pursue. Unknown as yet is the tendency for the phosphorothioate to approach other actively transcribing genes. These active transcription sites may undermine the in vitro inhibition that we observed. The tendency of these oligonucleotides to avoid positively charged "traps" or enzymes remains unknown and will be the subject of future investigations.

7. Targeting 1,10-phenanthroline scission with DNA-binding proteins

Finally, the attachment of 1,10-phenanthroline cuprous chelate ($[(OP)Cu]^+$) to proteins will be briefly discussed. Two strategies are possible in this approach. In one, we simply used a bridge ligand to couple the OP moiety to the protein.[20] In another, cysteine mutants of proteins were prepared and have been found to be extremely useful in mapping the binding sites of these proteins. These proteins include the Cro protein,[21] the Trp repressor (TrpR),[20, 22] the factor for inversion stimulation (Fis) of *E. coli*, and the Engrailed protein of *Drosophila*.[23–25]

DNase I footprinting provides a sensitive approach for localizing the binding site(s) of a protein by using it to block DNA hydrolysis. This technique of course has been enormously successful, but fails to provide one important result: No phase of the experiment indicates which amino acids make crucial contacts with the nucleic acid. These results are provided primarily by X-ray crystallographic studies which have shown that head-to-head binding is essential for the high-affinity binding of TrpR to its operator. These findings are supported by the targeted scission of the *trp* EDCBA promoter by a Trp repressor double mutant (A77S/E49C) in which 5-(iodoacetamido)-1,10-phenanthroline (**1**) is covalently linked to the protein through the sulfhydryl group at sequence position 49.

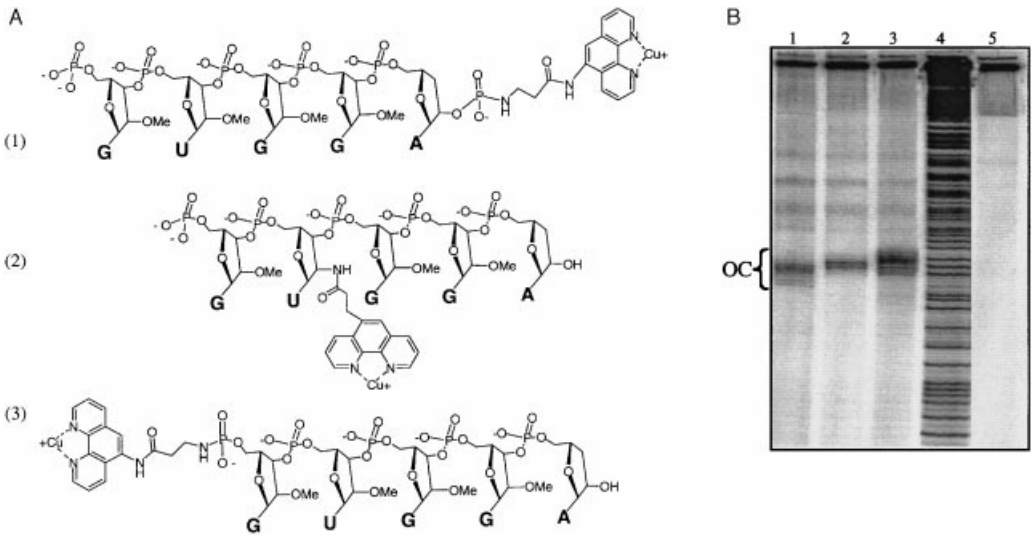
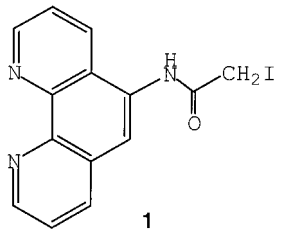


Figure 5. *Oligonucleotide structures and scission results. A) The three oligonucleotides used to infer the structure of the inhibited complex. The phenanthrolines have been placed at the 3′ end (1), in the interior on a uridine (2), and on the 5′ end of the RNA analogue of GUGAA (3). B) The scission by the oligonucleotide labeled in the interior with 2 – 3 base pair staggers is bracketed by the 3′-labelled oligonucleotide that cleaves 3′ to this scission site and the 5′-labelled oligonucleotide which cleaves 5′ to the scission site. Lane 1:* lac *UV5 scission by 30 μM 5′-GUGGA-3′ – OP; lane 2:* lac *UV5 scission by 30 μM 5′-GU-(OP)-GGA-3′; lane 3:* lac *UV5 scission by 15 μM OP – 5′-GUGGA-3′; lane 4: G + A ladder; lane 5:* lac *UV5 only. (Reprinted with permission from* Proc. Natl. Acad. Sci USA ***2000***, *97, 3136 – 3141. Copyright 2000 National Academy of Sciences, U.S.A.)*



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This operator has three binding sites for the repressor. We found that mixing the native (i.e. unmodified) Trp repressor with the *Sal* 1-cut DNA even in the excess of the modified repressor protein made double-stranded nicks in its target DNA as measured on a nondenaturing gel. We infer that these three binding sites are sufficient to sequester the 1,10-phenanthroline-modified repressor even as the concentration of protein is increased. Since the rate of repressor exchange is slow, the only scenario that can account for this result is the modified protein acting head to head to cleave the DNA without dissociation. Head-to-tail binding would not generate the observed results. As expected, the presence of tryptophan is essential for high-affinity binding; in its absence, the modified repressor protein makes random cuts in the target DNA (Figures 6 A and B).

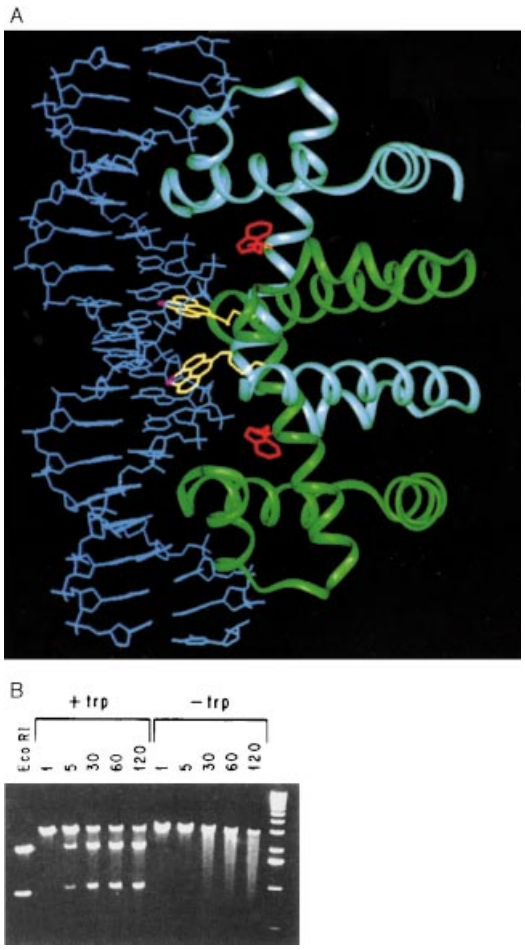

Figure 6. *Structure of Trp repressor – trp EDCBA operator DNA complex and cleavage pattern. A) The structure of the protein – DNA complex is presented. The protein has been modified by 5-iodoacetamido-1,10-phenanthroline. The binding sites for the tryptophan ligand are indicated. B) Time course of plasmid scission in solution with and without tryptophan. Linearized* trp *EDCBA-containing pUC vector (0.1 pmol) was cut with approximately 0.1 pmol of TrpR(A77S/E49C)-OP in a final reaction volume of 40 μL. Binding buffer was based on 50 mM Tris, pH 7.5, and contained tryptophan (0.5 mM final concentration) for lanes 2 – 6. The reaction was stopped at different time points ranging from 1 – 120 min as indicated by black numbers. (Reproduced with permission from* Protein Eng. ***1996***, 9, *603 – 610. Copyright 1996 Oxford University Press.)*

8. Conclusion

Our major accomplishment summarized in this article has been the description of the first oxidative nuclease and the demonstration of its mechanism of action. In this brief article, we have focused on the scission carried out by oligonucleotides as well as explored the reactivity of the 1,10-phenanthroline – copper(I) complex. We have shown that the open complex formed during transcription is a hybridizable structure that binds optimally to RNA-like 5-mers. We are now ready to develop methods that will transport the oligonucleotides into cells.

We have also shown that 1,10-phenanthroline – protein conjugates are efficient nucleases. The attachment of the 1,10-phenanthroline moiety to cysteine residues contained in DNA-binding proteins converts them into potent scission reagents. We have presented our results obtained with the TrpR, but the phenomenon of protein-targeted DNA scission has been observed in a number of proteins including the Cro protein of lambda phage, factor for inversion stimulation (Fis) of *E. coli*, and the Engrailed protein of *Drosophila*.

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