Chemical Nuclease Activity of 1,10-Phenanthroline−Copper. Isotopic Probes of Mechanism

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The chemical nuclease 1,10-phenanthroline−copper cleaves DNA by oxidative attack on the deoxyribose moiety yielding 3′- and 5′-phosphomonoesters, free purine and pyrimidine, and 5-methylenefuranone as stable products. Kinetic isotope effects associated with deuterium substitution have been measured in an attempt to analyze the chemical mechanism of the scission reaction. A kinetic isotope effect of 2.7 is observed with completely perdeuterated DNA, which is substituted in the oxidatively sensitive deoxyribose moiety as well as in the bases. Surprisingly, no isotope effect is found upon cleavage of DNA deuterated in the thymidines at either C-1′, C-2′,2″, or C-4′, all positions from which hydrogen is lost during the course of the reaction, by either the 2:1 or the 1:1 1,10-phenanthroline−cuprous complexes. These results suggest that perdeuteration of DNA alters the ligand binding and/or conformational flexibility of the nucleic acid.

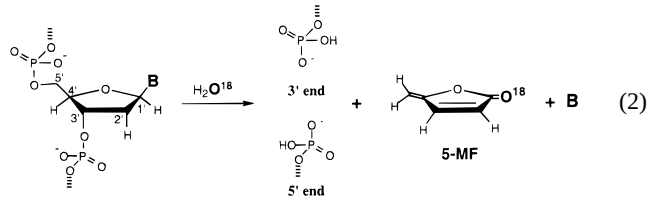
Introduction

The chemical nuclease 1,10-phenanthroline−copper (OP-Cu) cleaves DNA and RNA both as the 2:1 1,10-phenanthroline−cuprous complex ($(OP)_2Cu^+$) and as the 1:1 1,10-phenanthroline−cuprous complex linked to a ligand with high affinity for nucleic acids.[1,2] In each case, hydrogen peroxide is an essential coreactant.[3,4] The oxidative species formed from H_2O_2 and $(OP)_2Cu^+$ (and presumably from the tethered 1:1 complex) is not a diffusible species, such as hydroxyl radical or a freely diffusible chelate, and therefore the reaction must proceed through a noncovalent intermediate (eq 1). The binding affinity

$$(OP)_2Cu^+ + DNA \underset{}{\overset{K_d}{\rightleftharpoons}} (OP)_2Cu^+\text{---}DNA \xrightarrow[k_{cat}]{H_2O_2} \text{scission products} \qquad (1)$$

and orientation of the chelate relative to the oxidatively sensitive minor groove determine the cleavage specificity.

The principal products of the reaction generated by $(OP)_2Cu^+$ are 3′- and 5′-phosphomonoesters, free purine and pyrimidine, and 5-methylenefuranone (2).[5,6] The oxygen source of the



(2)

carbonyl group in 5-methylenefuranone (5-MF) is water.[7] A secondary reaction, presumably involving oxidative attack at the C-4′ hydrogen, leads to the production of a 3′-phosphoglycolate end and a three-carbon fragment which has not yet been identified.[8]

$(OP)_2Cu^+$ has been used as a footprinting reagent and as a probe of DNA and RNA secondary structure.[9−11] This chelate and closely related analogues are unique in their affinity for the single-stranded DNA formed at the active sites of prokaryotic and eukaryotic RNA polymerases during transcription initiation.[12−18] Redox inactive isosteres are effective inhibitors of both prokaryotic and eukaryotic promoters.[13,18] Ligands which have been used to target the 1:1 OP-Cu^+ complex have

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included oligonucleotides, DNA-binding drugs, and proteins.[19] The highly efficient and specific DNA scission directed by the *Escherichia coli trp* repressor and Fis proteins has provided useful insights into the binding orientation of these proteins to DNA.[20,21] Targeted scission of open complexes formed between RNA polymerase and DNA promoters by OP-Cu^+ linked to oligonucleotides complementary to the sequence at the initiation site of transcription has demonstrated their site-specific binding to the single-stranded template formed within the open complex.[22,23]

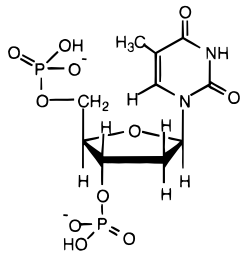
Structural information obtained from chemical cleavage experiments increases if the underlying mechanism of cleavage of the phosphodiester backbone is known. Since we sought to develop a method useful for analyzing nucleic acid scission by both the 2:1 and 1:1 phenanthroline−copper complexes, we have investigated the use of kinetic isotope effects (kie's) obtained with DNAs prepared from nucleotides composed of specifically substituted deuterated deoxyriboses. The experimental value of this method is that it would permit a direct comparison of the mechanism of cleavage by the chelates of different stoichiometries. Kinetic isotope effects have been informative in studying the cleavage of DNA by bleomycin[24] and neocarzinostatin,[25] but their applicability to the 1,10-phenanthroline−copper reaction has not been established.

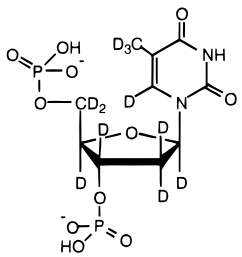
There are two approaches for studying kinetic isotope effects for reaction systems, such as $(OP)_2Cu^+$ or a bona fide enzyme, which involve the initial, reversible binding of the catalyst with its substrate followed by a bond-making/bond-breaking step. In the first approach, the reversible complex is quantitatively formed and the catalyst is fully committed to the forward direction of the reaction.[26] In this case, the isotope effects on the turnover number or maximal velocity (V_{max}) are examined. If the reaction is a multistep process and an elimination reaction were rate-limiting, the step involving hydrogen abstraction would be masked. The second approach for measuring isotope effects gauges the commitment to catalysis. In this case, the influence of isotopic substitution on the ratio of V_{max} to K_m would be determined. This can be written as

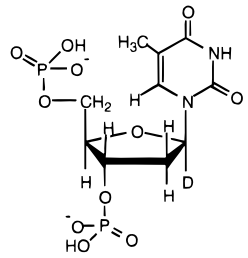
$$^{D}(V/K) = (k_h/k_d)((k_d + k_x)/(k_h + k_x)) \qquad (3)$$

where k_h and k_d are the rate constants for breaking the H and D bonds, respectively, while k_x represents all other processes which are nonproductive including the dissociation of the catalyst from the substrate (*i.e.*, $k_x = k_{dissociation} + k_{self-destruction}$) (3). If k_x is large relative to k_h and k_d, an isotope effect will be observed. If k_x is small, reflecting a high commitment to the forward direction, no isotope effect is observed. A kinetic isotope effect can be observed in the V_{max} case and not necessarily in $^{D}(V/K)$ unless it precedes or is the first irreversible step.

Since hydrogens from C-4′, C-2′, and C-1′ are all lost in the formation of 5-methylenefuranone and presumably also in the reaction leading to the minor product, 3′-phosphoglycolate, we synthesized thymidine derivatives with deuterium substituted at these positions. Unexpectedly, we find no measurable isotope


normal thymidine


perdeutero-thymidine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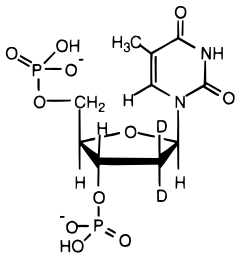
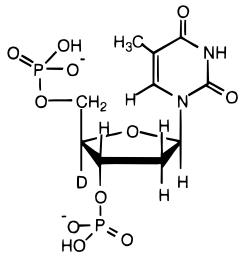

C-2',2"-deutero-thymidine


C-4'-deutero-thymidine

effect with deuterium substitution at any of these positions, although a substantial isotope effect was measured when perdeuterated DNA isolated from bacteria grown in completely deuterated media was used as a substrate. The origin of the isotope effect observed with the perdeuterated DNA, in which the bases as well as the riboses are substituted at the nonexchangeable positions, is unknown, although substantial secondary/steric deuterium isotope effects are observed in other systems.

Materials and Methods

Preparation of Perdeuterated DNA. The plasmid pBVM (constructed from pUC18 and pHC314) containing the *lac* UV5 promoter was transformed into competent *E. coli* DH5 α cells.[27] Fully deuterated DNA was produced by growing the bacteria in deuterated medium, Celtone D (Martek Bioscience Corporation). The transformed bacteria were adapted for the growth in the deuterated medium. This was accomplished in steps of 70%, 90%, and 99.8% D_2O enrichment. At each of these levels, *E. coli* cells were grown in Luria−Bertani (LB) medium prepared with water containing the appropriate percentage of D_2O, and ampicillin was added to a concentration of 200 μg/mL. The bacteria were incubated with shaking at 37 °C for 18 h ($A_{600} \geq 1$). An aliquot was transferred to an appropriately

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deuterated agar plate and incubated at 37 °C. Single colonies were then isolated and grown at the next higher level of D_2O enrichment. After adaptation to 99.8% D_2O, the bacteria were grown in fully deuterated medium Celtone D containing ampicillin (200 μg/mL) and thiamine (0.01%). The plasmid was isolated using a protocol provided by Quiagen and digested with Eco*RI* and Pvu*II* to generate a 186-bp fragment containing the *E. coli lac* UV-5 promoter (sequence positions −123 to +63). As an internal standard a double-stranded fully protonated oligonucleotide of the following sequence was ligated to both the fully protonated and fully deuterated restriction fragments:

5'-pAATTGCTAAGCTCATCCGGTACGT-3'

3'-CGATTCGAGTAGGCCATGCA^{33}p-5'

The ligation product was purified by PAGE (12%) under nondenaturing conditions. The correct sequence was verified by dideoxy sequencing of the nontemplate strand.

Chemical Syntheses of DNA Deuterated Specifically at C-1′, C-2′,2″ or C-4′ in All the Thymidines. Three thymidine triphosphates deuterated at C-1′, C-2′,2″, or C-4′ and one thymidine phosphoramidite deuterated at C-1′ were synthesized according to published procedures (general methods;[28,29] deuteration at C-1′;[30] deuteration at C-2′,2″;[31] deuteration at C-4′ [32]). The correct structures of the compounds were verified by ^{1}H NMR. The deuterium content, determined from the ^{1}H NMR spectra of the triphosphates and phosphoramidite, was 95%.

The C-1′ or C-2′,2″ and C-4′ deuterium labeled thymidine triphosphates were incorporated into DNA by PCR using the 186-bp *E. coli lac* UV-5 promoter as template. The nontemplate strand primer corresponded to the promoter sequence from −96 to −77, and the template strand primer, 5′-end-labeled with ^{33}P, corresponded to the sequence from +36 to +63. The resulting 159-bp PCR products, containing the *lac* UV-5 promotor from sequence positions −96 to +63, were purified by PAGE (8%) under nondenaturing conditions.

Several *lac* UV-5 promoter mutants (fully protonated) containing a T at one of the positions −3, −4, or −6 instead of the wild-type base were also synthesized by PCR using template primers containing the desired mutations.[33] The purified PCR products containing these mutations were then used as templates for PCR reactions in which the protiothymidine triphosphate was replaced by the C-1′ deuterium labeled thymidine triphosphate to generate three 159-bp promoter mutants (labeled A3T, C4T, or C6T in Table 3).

The C-1′ deuterated thymidine phosphoramidite was incorporated into DNA (sequence: −40 to +40 of the *lac* UV-5 promoter) by chemical methods, purified, and labeled with ^{33}P.

Cleavage of the Duplex DNA Substrates by $(OP)_2Cu^+$ and Analysis of the Cleavage Products. Cleavage of protonated and deuterated DNA substrates by $(OP)_2Cu^+$ was performed in Tris·HCl buffer (50 mM, pH = 8.0) at 37 °C using 10:1 1,10-phenanthroline/$CuSO_4$ and 3-mercaptopropionic acid (MPA, 5.8 mM) as the reducing agent under conditions where less than 10% of the total labeled DNA substrate was consumed. In the case of perdeuterated DNA, the concentration of $(OP)_2Cu^+$ (based on that of $CuSO_4$) was 10 μM and the concentration of the DNA was 200 nM (nucleotides: nts). In the case of DNA deuterated specifically at C-1′, C-2′,2″, or C-4′ of all the thymidines, the concentrations of $(OP)_2Cu^+$, DNA, and added exogenous H_2O_2 and the order of addition of the reagents (i.e., whether MPA or preformed $(OP)_2Cu^+$ was added last to initiate the cleavage reaction) are given in Table 2. Reaction times varied from 30 to 120 s, and the reactions were stopped by addition of an excess of 2,9-dimethyl-1,10-phenanthroline (300−1000-fold over Cu) and extracted with chloroform. The aqueous phase was diluted with loading buffer (80% formamide, 0.01% bromophenol blue, 0.01% xylene cyanol), heated to 90 °C for 5 min, and loaded onto a 20% denaturing polyacrylamide gel. After electrophoresis, the gel was dried on Whatman filter paper.

Table 1. Kinetic Isotope Effects for Cleavage of Perdeuterated DNA by $(OP)_2Cu^+$

sequence position	kie expt 1	kie expt 2	mean
T_{51}	2.9		
G_{52}	2.8		
C_{54}	2.9	3.4	3.2 ± 0.4
T_{55}	2.4		
A_{56}		2.7	
A_{57}	2.5		
G_{58}		2.9	
T_{59}	2.4	2.8	2.6 ± 0.3
G_{60}		2.4	
A_{61}		2.7	
T_{65}	2.0	2.2	2.1 ± 0.1
A_{66}		2.1	
mean	2.6 ± 0.3	2.7 ± 0.4	

The quantitation of the bands was performed on a Molecular Dynamics PhosphorImager using the program ImageQuant. In the case of scission of the perdeutero DNA, the magnitude of the kinetic isotope effect was determined by a comparison of the normalized band intensities at a specific cleavage site of protonated and of deuterated DNA (Figure 1 and Table 1). A normalization factor was obtained by comparing the band intensities of the protonated linker regions from lanes containing protonated and deuterated DNA. In the case of scission of the DNA specifically labeled with deuterium at a single deoxyribose carbon, the kinetic isotope effect was determined by comparing the normalized intensities of the strong cutting sites at T_{26} and T_{24} for both the protonated and deuterated DNA. Here, a normalization factor was obtained by comparing the intensities at the sites G_{25} or G_{23} in lanes containing unlabeled DNA with those in lanes containing deuterium-labeled DNA.

Cleavage of the *E. coli* RNA Polymerase−*Lac* UV-5 Promoter Open Complex Deuterated at C-1′ in All the Thymidines. Each ^{33}P-labeled *lac* UV-5 promoter mutant (∼200 fmol) was incubated with *E. coli* RNA polymerase (Pharmacia, ∼2 pmol) in 10 μL of buffer (40 mM Tris·HCL, pH 8, 50 mM KCl, 10 mM $MgCl_2$) at 37 °C for 20 min. Next, 1 μL of a freshly prepared stock solution of (5-ϕOP)$_2$Cu$^+$ (5-ϕOP = 5-phenyl-1,10-phenanthroline; 840 μM 5-ϕOP/400 μM $CuSO_4$) in 95:5 water/ethanol was added. The cleavage reaction was initiated by the addition of 1 μL of 58 mM MPA, and the reaction was quenched after 4 min by adding 3 μL of 40 mM 2,9-dimethyl-1,10-phenanthroline in ethanol. The reactions were then treated as described above.

Targeted Scission of Single-Stranded DNA by an Oligonucleotide−1,10-Phenanthroline−Copper(I) Chimera. An oligonucleotide corresponding to the sequence of the nontemplate strand of *lac* UV-5 from positions +17 to +40 was

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Table 2. Kinetic Isotope Effects for $(OP)_2Cu^+$ Cleavage of Duplex DNA Deuterated at C-1′, C-2′,2″, or C-4′ of All the Thymidines

entry	DNA	location at D	[DNA] (μM in nts)	[H_2O_2] added (mM)	[$(OP)_2Cu^+$] (μM)	initiatn by addn of:	kie
1	*lac*UV5[a]	C-1′	0.2−0.6	0	10	MPA	1.0 ± 0.1[c]
2	*lac*UV5[a]	C-1′	0.2	8.8	1	$(OP)_2Cu^+$	1.1 ± 0.4[c]
3	*lac*UV5[b]	C-1′	3−6	0	10	MPA	0.98
4	*lac*UV5[b]	C-1′	480	8.8	1	$(OP)_2Cu^{2+}$	0.93 ± 0.01[d]
5	*lac*UV5[b] + ct DNA	C-1′	1000	0	10	$(OP)_2Cu^{2+}$	0.98 ± 0.17[d]
6	*lac*UV5[a]	C-2′,2″	0.2	0	10	MPA	1.0
7	*lac*UV5[a]	C-2′,2″	0.2	8.8	1	$(OP)_2Cu^{2+}$	1.0
8	*lac*UV5[a]	C-4′	0.2	0	10	MPA	1.0 ± 0.06[e]

[a] DNA prepared by PCR (159-bp *lac* UV-5 fragment). [b] DNA prepared by chemical synthesis (80-bp *lac* UV-5 fragment). [c] Six bands (cut sites) were examined. [d] Two bands were examined. [e] Four bands were examined.

Table 3. Kinetic Isotope Effects for (5-ϕOP)$_2Cu^+$ [a] Cleavage of the *E. coli* RNA Polymerase−*lac* UV-5[b] Open Complex Deuterated at C-1′ of All the Thymidines

entry	DNA[c]	[H_2O_2] added (mM)	initiatn by addn of:	kie
1	C6T*lac*UV5	0	MPA	1.05
2	A3T*lac*UV5	0	MPA	1.1
3	C4T*lac*UV5	0	MPA	1.2 ± 0.2[d]
4	C6T*lac*UV5	8.8	(5-ϕOP)$_2Cu^+$	1.3−1.4
5	A3T*lac*UV5	8.8	(5-ϕOP)$_2Cu^+$	1.0
6	C4T*lac*UV5	8.8	(5-ϕOP)$_2Cu^+$	1.0

[a] 50 μM (5-ϕOP)$_2Cu^+$. [b] [DNA] = 3−12 μM in nts. [c] *Lac* UV-5 promoter mutants in which the first specified base at the specifed number of positions before the first transcribed base has been replaced by a thymidine. [d] Two bands were examined.

synthesized, 5′-labeled with thiophosphate, derivatized with5-iodoacetamido-1,10-phenanthroline, and purified as described previously.[34]

OP-NHCOCH$_2$S-P(O)$_2$-O-CAATTTCACACAGGAAACAGCTAT-3'

Two single-stranded targets, corresponding to the template strand of *lac* UV-5 from −40 to +40, were labeled with ^{32}P at their 5′-ends. One was fully protonated DNA, while the other was synthesized using C-1′-deuterium-substituted thymidine phosphoramidate. For the cleavage experiment, the single-stranded 80-mer (1.5 fmol) and the OP-derivatized oligonucleotide (0.43 pmol) in 18 μL of buffer (50 mM, pH 7.9 Tris/50 mM NaCl/ 1mM $MgCl_2$) was heated to 90 °C for 5 min and then allowed to anneal at room temperature for 30 min. The cleavage reaction was initiated by addition of aqueous $CuSO_4$ (1 μL, 100 μM) and MPA (1 μL, 100 mM). After 2 h at 37 °C, the reaction mixture was quenched with neocuproine. The cleavage products were analyzed on a 15% denaturing polyacrylamide gel.

Results

Kinetic Parameters of the DNase Activity of $(OP)_2Cu^+$. The goals of these studies were (a) to identify the rate-limiting step in the multistep pathway for the scission of DNA using kinetic isotope effects and (b) to establish if the same steps controlled the overall reaction when both the 2:1 and 1:1 complexes of OP and copper were used. Our initial objective therefore was to determine the kinetic constants for this multicomponent system in order to establish the conditions required for the determination of $^D(V/K)$ and ^DV.

In our earlier studies, we demonstrated that H_2O_2 was an essential coreactant by inhibiting the cleavage reaction with catalase[3] and showing that exogenously added H_2O_2 in the millimolar concentration range significantly enhanced the rate of scission.[35] We have generally carried out the cleavage of DNA by the 2:1 complex using H_2O_2 generated in situ for experimental convenience and because of the instability of low concentrations of peroxide. We have now determined the concentration dependence of the reaction on hydrogen peroxide to be sure that the generation of hydrogen peroxide is not the rate-determining step under our cleavage conditions. We find that the K_m of hydrogen peroxide is 10 μM when the concentra-

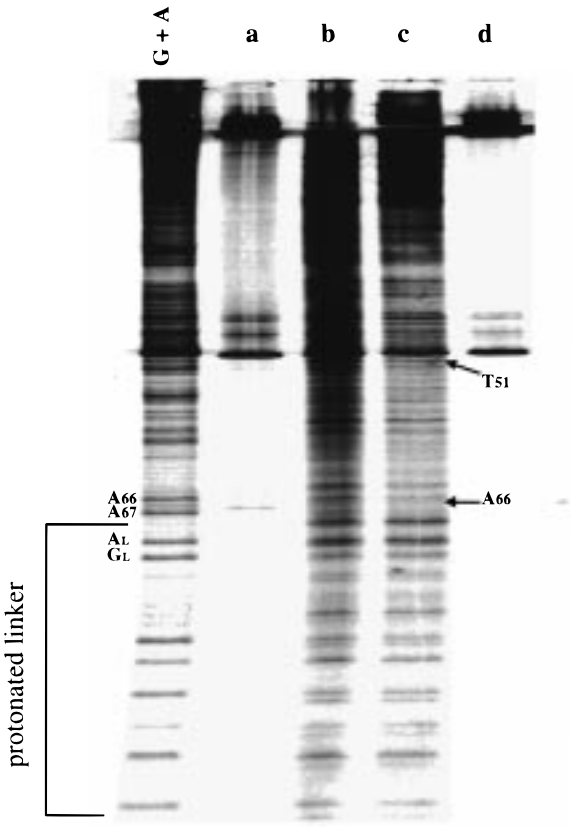

Figure 1. Cleavage of the perdeuterated *lac* UV-5 DNA chimera by $(OP)_2Cu^+$. The first base of the *lac* UV-5 DNA following the protonated linker region is assigned A_{67}. G + A lane: Maxam−Gilbert G + A sequencing ladder. Control lanes a and d: untreated protonated and deuterated chimeric DNA, respectively, with the template strand 5′-end-labeled with ^{33}P. Lane b: cleavage of the *protonated* chimeric DNA (0.2 μM nts) by $(OP)_2Cu^+$ (10 μM $CuSO_4$/100 μM OP) for 1 min at 37 °C. Scission was started by addition of MPA (5.8 mM) and quenched with 2,9-dimethyl-1,10-phenanthroline (4 mM). Lane c: cleavage of the *deuterated* chimeric DNA by $(OP)_2Cu^+$ under identical conditions. The kinetic isotope effects were determined by comparison of the band intensities at sequence positions $A_{66}-T_{51}$ in lane b with those in lane c after normalization using the protonated linker regions.

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tion of the chelate is 10 μM (100 μM OP, 10 μM Cu^+). Since the ambient solubility of oxygen is 200 μM, H_2O_2 generation in situ is not likely to be rate-limiting. In addition, we have measured the K_m for DNA for the scission of the *lac* UV-5 sequence at positions $T_{26}-G_{23}$ by diluting ^{33}P-labeled *lac* UV-5 DNA with calf thymus (ct) DNA and assaying the scission (100 μM OP, 10 μM Cu^+, H_2O_2 generated in situ). The estimated K_m was 200 μM. As a result, cleavage reactions with $(OP)_2Cu^+$ to measure kinetic isotope effects were carried out at DNA concentrations at or below this K_m except for entries 4 and 5 in Table 2.

Kinetic Isotope Effects for Perdeuterated DNA. The chemical mechanism of DNA scission by $(OP)_2Cu^+$ is a multistep reaction with many potential points of rate limitation.[7] Prior to examining the effects on the kinetics of isotope substitution at each one of the positions possibly participating in a rate-determining step, we prepared perdeutero DNA biologically. The methodology outlined in Materials and Methods provided a discrete fragment of the *lac* UV-5 promoter which was deuterated not only at all the nonexchangeable C−H bonds of the deoxyribose units but also at those of the purines and pyrimidines. The motivation for preparing this DNA was to determine if any isotope effect could be observed in the system prior to undertaking the labor intensive task of regiospecific synthesis of specifically deuterated thymidines.

To assay the magnitude of the kie, a fully protonated oligonucleotide with the following sequence was ligated to both the deuterated and fully protonated *lac* UV-5 EcoR*I* fragment:

5′-pAATTGCTAAGCTCATCCGGTACGT-3′

3′-CGATTCGAGTAGGCCATGCA^{33}p-5′

The data reported in Table 1 and Figure 1 indicates that under *V*/*K* conditions measurable isotope effects of approximately 2.7 are observed. An important aspect of these results is that the observed kie's are not nucleotide dependent. Kie's occur at cleavage sites of T, G, C, and A, and the magnitudes are the same within experimental error. The results with the perdeuterated DNA indicated to us that isotope effects would likely be observed with DNAs specifically labeled in the thymidine residues, so their syntheses were undertaken. This nucleotide was chosen for modification because of synthetic simplicity. *The assumption in these studies was that the observed isotope effect would be attributable to a deuterium isotope effect associated with one of the hydrogens that was lost during the formation of the 5-methylenefuranone product.*

Kie's with DNAs Prepared from Monodeuterated Thymidine. (A) C-1′-Deuterium-Substituted Deoxyribose. Four distinct kie's were measured using DNAs prepared from specifically monodeuterated thymidines. The most extensive set of experiments were carried out on C-1′-deuterium-substituted (C-1′-D) thymidines. In the first series of experiments, PCR amplified DNA derived from the *lac* UV-5 operon was cleaved under a variety of conditions. The features of the reaction system which were systematically varied included concentrations of DNA, chelate, hydrogen peroxide, and thiol and the order of addition of the reactants. For example, in Table 2, entries 1 and 2, the kie was independent of the order of addition of the reactants and of the addition of hydrogen peroxide. The striking feature of the results summarized in Table 2 (see also Figure 2) is that no isotope effect was observed within our experimental limits with C-1′-deuterium- labeled thymidines despite the readily observed kie with perdeutero-DNA and the obligatory removal of this hydrogen during the formation of 5-methylenefuranone.

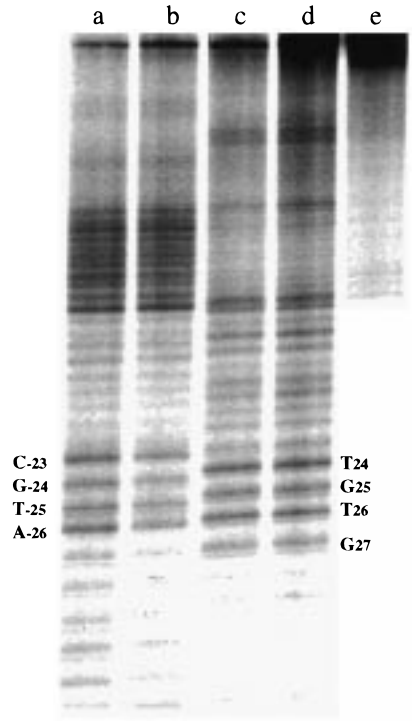

Figure 2. Cleavage of C-1′-deuterated *lac* UV-5 DNA by $(OP)_2Cu^+$. Lane a: cleavage of the ^{33}P-labeled nontemplate strand of DNA (0.2 mM nts) containing C-1′-deuterated T's by $(OP)_2Cu^+$ (10 μM $CuSO_4$/100 μM OP) for 1 min at 37 °C. Scission was started by addition of MPA (5.8 mM) and quenched with 2,9-dimethyl-1,10-phenanthroline (4 mM). Lane b: cleavage of the ^{33}P-labeled nontemplate strand of fully protonated DNA. Lane c: cleavage of the ^{33}P-labeled template strand of DNA containing C-1′-deuterated T's. Lane d: cleavage of the ^{33}P-labeled template strand of fully protonated DNA containing C-1′-deuterated T's. Lane e: untreated fully protonated DNA with the template strand ^{33}P-labeled at the 5′-end. Similar controls with the nontemplate strand labeled and with DNA containing C-1′-deuterated T's are not shown.

C-1′-deuterium-labeled DNA was also used to determine whether an isotope effect could be observed for cleavage of the open complex formed between RNA polymerase and the *lac* UV-5 promoter. The scission of the open complex on the template strand is a unique reactivity of $(OP)_2Cu^+$ and closely related analogues and presumably arises from the preferential binding affinity of the tetrahedral cuprous chelates for this essential intermediate in transcription. The open complexes formed with several *lac* UV-5 promoters with a T inserted in place of the wild-type nucleotide at sequence positions −3, −4, or −6, sites of stong cleavage by the chelates, were subjected to scission by $(5\text{-}\phi OP)_2Cu^+$. The results summarized in Table 3 indicate that no isotope effect is observed in the scission at T within the open complex.

(B) C-2′-Deuterium-Substituted Deoxyribose. DNAs derived from the *lac* UV-5 promoter were also prepared with thymidine specifically deuterated in the C-2′,2″ position. In our studies demonstrating that water is the atom source of the oxygen incorporated into 5-methylenefuranone, we proposed that a stabilized allylic cation may be formed in the reaction pathway.[7] This carbocation could result from initial hydrogen abstraction at the C-1′ position of the sugar moiety and subsequent oxidation, *or* by initial abstraction of the C-2′ hydrogen followed by oxidation to the C-2′ carbocation and subsequent 1,2 hydride migration to form the C-1′ carbocation, as shown by Saito et al.[36]

To determine whether the C-2′,2″ position of the sugar moiety was associated with a slow step in the reaction, we synthesized the dideuterothymidine. Our kinetic data clearly indicate that no isotope effect is observed under $^{D}(V/K)$ conditions for C-2′,2″-D DNA (Table 2, entries 5 and 6). Neither hydrogen abstraction nor proton loss from C-2 is associated with a rate-limiting step.

(C) C-4′-Deuterium-Substituted Deoxyribose. Our study of the primary kinetic isotope effects also included investigation of the scission of *lac* UV-5 DNA prepared using PCR with C-4′-D thymidine triphosphate. Substitution at this position is of interest because it was shown that 3′-phosphoglycolate, which is most likely the result of initial abstraction of the C-4′ hydrogen, is also a product of cleavage by $(OP)_2Cu^+$ and may contribute to the unresolved band observed on a sequencing gel. Cleavage of this DNA again revealed no measurable isotope effect (Table 2, entry 8). Since we could not resolve the bands on a high-resolution gel, we cannot exclude a minor contribution of the C-4′ pathway to the overall reaction which might partially contribute to the observed kie in the experiment using perdeutero-DNA as a substrate.

(D) Lack of Isotope Effect in Targeted Scission. Cleavage by the tetrahedral chelate involves reversible binding in the minor groove of DNA as the first step. In contrast, targeted scission by 1:1 OP-Cu linked to either a protein or a nucleic acid involves prior binding of the conjugate to the target and subsequent activation of the cleavage reaction by addition of a reducing agent under aerobic conditions.[19,37] Although hydrogen peroxide is required in these reactions, the rate of targeted scission is not enhanced by addition of exogenous H_2O_2, in contrast to the reaction of the 2:1 tetrahedral complex. Isotope effects for targeted scission reactions would therefore provide information on the initial hydrogen abstraction and subsequent rearrangement (i.e., k_{cat}, eq 1) during the cleavage reaction. Therefore, we examined the scission of 5′-^{33}P-labeled single-stranded DNA (*lac* sequence from −40 to +40) prepared with thymidines deuterated at C-1′ by a complementary oligonucleotide (*lac* sequence from +17 to +40) derivatized at the 5′-end with OP. Typically, two or three sites of scission are observed under these circumstances, and it is possible to evaluate an isotope effect when cleavage of the deuterated strand is carried out in parallel with cleavage of the nondeuterated strand of identical sequence. In Figure 3, the two strongest sites of cleavage are at G_{17} and T_{16}, which corresponds to the transition from double-stranded to single-stranded DNA. After normalization using the bands at G_{17}, the intensities of the two bands at T_{16} (lanes c and d) were found to be identical. Our results clearly indicate that no isotope effect is observed under these conditions.

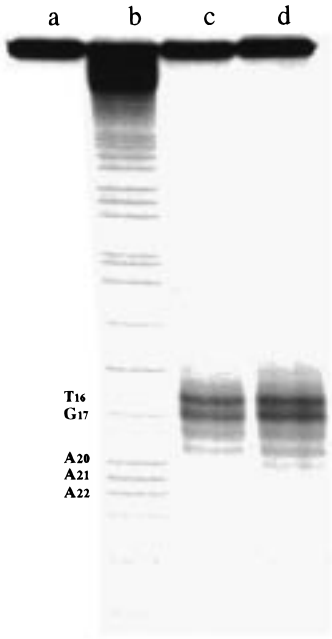

Figure 3. Targeted scission of single-stranded DNA by an oligonucleotide−1,10-phenanthroline−copper(I) chimera. Lane a: single-stranded fully protonated DNA (*lac* UV-5 template strand sequence from −40 to +40) labeled at the 5′-end with ^{33}P. Lane b: Maxam−Gilbert G + A sequencing ladder of the same 80-mer DNA. Lane c: cleavage of the fully protonated 80-mer after hybridization to the OP-derivatized oligonucleotide (*lac* UV-5 nontemplate strand sequence from +17 to +40). Lane d: cleavage of the 80-mer containing C-1′-deuterated T's after hybridization to the OP-derivatized oligonucleotide.

Discussion

Our investigation of kinetic isotope effects of DNA scission by the OP-Cu system was motivated by its successful application to the study of DNA cleavage by bleomycin[24,26,38] and neocarzinostatin.[25] The goal was to use kie's to compare the mechanism of scission by the 1:1 and 2:1 1,10-phenanthroline−copper chelates. Our results have led us in an unanticipated direction. We have found readily measurable kinetic isotope effects of 2.7 ± 0.4 with perdeuterated DNA prepared biologically in which all the nonexchangeable hydrogens in both the deoxyriboses and bases have been substituted with deuterium (Table 1, Figure 1). However, when the deoxyribose of thymidine was systematically substituted at the C-4′, C-2′, and C-1′ positions and the deuterated nucleotide incorporated into DNA, *no* kinetic isotope effects were observed. This result was obtained when the 2:1 chelate was used to cleave B-DNA (Table 2) and the open complex formed with RNA polymerase and the *lac* UV-5 promoter (Table 3) as well as in targeted scission of single-stranded DNA by the 1:1 chelate upon hybridization of a complementary OP-linked strand (Figure 3).

No measurable isotope effects are observable whenever stereospecifically substituted DNAs are used as substrates. This result holds true whether the reaction is carried out under "nonsaturating" conditions (V_{max}/K_m, Tables 2 and 3) or under "saturating" conditions (k_{cat}, Figure 3) characteristic of targeted cleavage reactions in which the noncovalent complex is formed prior to activation by thiol addition. In the latter case, only the cleavage of C-1′-D DNA was examined. The curious feature of the kie's for the perdeuterated DNA cleaved by the tetrahedral complex is that they are independent of sequence position and nucleotide. This contrasts with the results obtained with bleomycin where different kie's are seen as a function of both sequence and nucleotide, possibly reflecting the sequence dependent conformational variability of DNA which has been observed in high-resolution structures of oligonucleotides.[39] No isotope effect in bleomycin cleavage was observed when DNAs prepared with C-2′-deuterated mononucleotides were used as substrates even though elimination is observed at this position as well as at C-4′.

The absence of a kinetic isotope effect for the cleavage of the C-4′-D, C-2′-D, and C-1′-D DNAs by $(OP)_2Cu^+$ indicates

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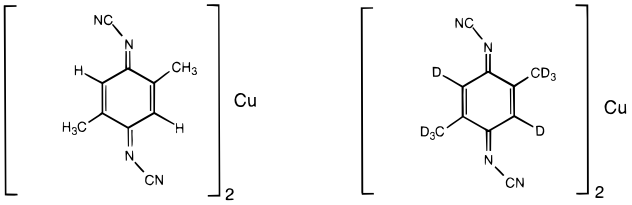
a *high* commitment to catalysis. In algebraic terms, that means k_x in eq 3, representing the sum of nonproductive processes, is small relative to k_h and k_d. As a result, no net kinetic isotope effect is observed upon deuterium substitution in the deoxyribose. Conversely, the measurable isotope effect of 2.7 with the perdeutero-DNA indicates that the commitment to catalysis is low since as k_x increases, the magnitude of the isotope effect increases. What feature of the perdeutero-DNA causes this marked change in the value of k_x? One possibility is that the perdeutero-DNA in which the bases and all the deoxyriboses are deuterated possesses different ligand-binding affinity and/or conformational flexibility than the DNAs prepared with specifically substituted deoxyriboses.

Previous studies in our laboratory have demonstrated that B-DNA is cleaved more efficiently than A-DNA.[40] The A structure used in that work was generated by hybridizing an RNA strand to a complementary DNA strand. Both strands were cleaved with comparable rates in the heteroduplex by $(OP)_2Cu^+$, but the rates of scission were 8−9-fold slower than those for the two strands in the B form. These findings suggest that the weaker binding of $(OP)_2Cu^+$ to the broad flat minor groove of the A structure is responsible for its diminished reactivity. We propose that these observations may be relevant to the measurable kie's observed with perdeuterated DNA. The kie of 2.7 with perdeuterated DNA indicates that the binary complex formed when $(OP)_2Cu^+$ binds to perdeuterated DNA exhibits a lower commitment to catalysis (higher k_x). One possible origin of this unusual isotope effect may be that the minor groove of the perdeuterated DNA is widened due to the lower zero-point vibrational energy of a C−D bond relative to a C−H bond. This would lead to a decreased rate of cleavage of deuterated DNA relative to normal DNA, as was observed in the comparison of scission of A- and B-DNA.

Literature precedents for steric isotope effects and ligand-binding effects associated with the perdeuteration of ligands include an isotope effect in the racemization of 9,10-dihydro-4,5-dimethylphenanthrene,[41] the diminished binding of deuterated benzene relative to benzene to the copper(II)−ethylenediamine type of tetracyanonickelate clathrate,[42] and an inverse isotope effect of 2.6 for the hydrolysis of perdeutero-*tert*-butyl chloride.[43] The latter effect may arise from either hyperconjugative or steric effects in a reaction which proceeds through a carbon-centered cation, as has been proposed in the cleavage of DNA by $(OP)_2Cu^+$.[7]

Perhaps the most intriguing example of a substantial secondary deuterium isotope effect is the dramatic alteration of conductivity in copper chelates of 2,5-disubstituted *N*,*N*′-dicyanoquinone diimines upon deuterium substitution.[44] The chelate of fully protonated 2,5-dimethyl-*N*,*N*′-dicyanoquinone diimine remains metallic even at the lowest achievable temperature (0.4 K). However, deuterium substitution raises the phase transition temperature to a semiconductor to 82 K. Significant effects are also observed in the ESR signals, magnetic susceptibilities, and X-ray structures. The 7-fold diamondoid superstructure, which includes infinite superhelices in the stacks of ligands and copper ions, exhibits significantly higher compressibility in the direction of stacking in the case of the deuterated chelates relative to the fully protonated chelate. Due to the lower zero-point energy, the mean as well as the maximum C−D bond lengths are less than those of the C−H bonds. Therefore, the helices in the superstructure of the deuterated salts form more compliant "springs" upon cooling. The differences in compressibility lead to differences in a certain bond angle which correlates with the conductive properties of the salts.



All deoxyribose directed oxidative DNA scission reactions are multistep processes. If reaction pathways of roughly equivalent kinetic barriers are available, deuterium substitution at either C′-1, C-2′, or C′-4 may channel the reaction down pathways which minimize isotopic barriers. In contrast, perdeuterated DNAs will be unable to proceed by a pathway unaffected by isotopic substitution. In addition to possible rate limitations reflecting altered ligand binding and groove conformation, this may result in an enhanced isotope effect.

A partially rate limiting formation of the oxidative species responsible for initiating the cleavage reaction may also influence the magnitude of the observed isotope effect. Although the structure of the oxidative species formed by the reaction of H_2O_2 with the cuprous ion is the subject of active investigation and has not yet been determined, its rate of formation conceivably could be influenced by the extent of substrate deuteration. All these complexities indicate that the use of kie's to study a deoxyribose cleavage reaction may not be a reliable tool of mechanistic investigation despite its limited success with bleomycin and neocarzinostatin. With the nuclease activity of 1,10-phenanthroline−copper, our results indicate that no isotope effect is observed when either the 2:1 or 1:1 complex is used to cleave DNAs labeled at a single site.

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