Scission of RNA by the chemical nuclease of 1,10-phenanthroline-copper ion: preference for single-stranded loops

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ABSTRACT

The scission of RNA by the chemical nuclease activity of 1,10-phenanthroline-copper (OP-Cu) has been studied using a lac mRNA fragment and tRNA^{Phe} as substrates. Since the chemical mechanism of scission involves oxidative attack on the ribose, scission is observed at all nucleotides including dihydrouridine and Y-bases. Specificity for single-stranded loop regions is apparent from the similarity of the reactivity of OP-Cu to the single-strand specific reagents dimethyl sulfate and diethyl pyrocarbonate using the fragment of lac mRNA as a substrate. Similar preference is observed in the reaction with tRNA although scission in the helical acceptor stem is also observed.

INTRODUCTION

The 1,10-phenanthroline-cuprous complex with hydrogen peroxide as a coreactant has a nuclease activity which nicks both RNA and DNA (1-3). The chemical mechanism of DNA scission involves the oxidative attack on the C-1 of the deoxyribose followed by rapid elimination reactions leading to strand scission (4,5). In its reaction with DNA, the nuclease activity exhibits two aspects of specificity. The preferred secondary structure is B-DNA which is cleaved at three times the rate of A-DNA; Z-DNA is unreactive, while single-stranded DNA is a poor substrate (3,6). With DNA in the B-structure, the chemical nuclease activity exhibits sequence dependent reactivity without exhibiting base specificity at the site of cutting. Both the secondary structure and primary sequence preference are attributable to the formation of an essential noncovalent intermediate between the coordination complex and the DNA during the reaction.

RNA is also a substrate for the nuclease activity (3,7). When the RNA is constrained to the same conformation as the DNA, the phosphodiester bond of RNA is cleaved with comparable efficiency. For

example, in the heteroduplex formed between poly(rA) and poly(T), both strands are equally susceptible to scission (3). If a deoxyoligonucleotide is linked at the 5' end with 1,10-phenanthroline, the resulting reagent will cleave RNA and DNA of identical sequence with equivalent efficiency (7). Although the detailed mechanism of the elimination reactions associated with the ribose moiety of RNA must be different than those associated with the deoxyribose moiety of DNA, the oxidative mechanism for scission of both nucleic acids may share the same rate determining step.

The specificity of the unsubstituted 2:1 1,10-phenanthroline-copper complex in the scission of an RNA possessing stem-loop structures has not been previously examined. For *deoxyoligonucleotides* capable of forming stem-loop structures, the B-helix of the stem is more reactive than the loop (8). In this study, we demonstrate that OP-Cu shows a preference for single-stranded loops relative to double-stranded A-structures present in RNAs.

EXPERIMENTAL PROCEDURES

Preparation of lac mRNA

Plasmid pGM820, a plasmid derived from pGEM2 (Promega Biotec, Madison WI) and containing the 5' region of the *Escherichia coli lacZ* gene behind the pGEM2 vector's bacteriophage T7 promoter, was used as the template for in vitro RNA synthesis. The *lac* sequences encompass sequence positions -19 to + 63. After digesting the plasmid with *Sph*I and creating flush ends with the large fragment of DNA polymerase I, transcription was carried out using T7 RNA polymerase to generate a 554 nucleotide run-off transcript (9). The reaction mixture was then treated with DNase I, extracted with phenol and precipitated with ethanol, and the RNA was resuspended in distilled water at 0.1 pmol per μ l.

Preparation of 3'-Labeled lac mRNA

To prepare 3'-labeled RNA, the DNA template was digested with *Eco*R1 prior to transcription. *lac* mRNA was ligated to [32 P]pCp (Amersham) using T7 RNA ligase (BRL) (10). After the labeling reaction, the RNA were phenol/chloroform and chloroform extracted, passed through a G-50 spin columns and evaporated to dryness (Savant Speed-Vac). After resuspension in 80% formamide, 0.1% bromophenol blue and 0.1% xylene cyanol, the mRNA was loaded on a small 4% denaturing acrylamide gel, which was run at 200 V for 20 minutes. Individual

bands were cut out and eluted overnight at 37° in 0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS (11). Eluted mRNA was ethanol precipitated, resuspended in 50 mM Tris-HCl, pH 7.5 and stored at -20°.

OP-Cu Digest of 3'-Labeled lac mRNA

Labeled lac mRNA (50,000 cpm) was digested in either 50 mM Tris-HCl, pH 8.0 or 50 mM Tris-HCl, pH 8.0, 10 mM magnesium chloride, 50 mM NaCl with 50 μ M OP, 12 μ M CuSO₄ and 5.8 mM mercaptopropionic acid at room temperature for 2 to 5 min as indicated. Digestions were quenched with 2.1 mM 2,9-dimethyl OP. An equal volume of 10 M urea, 0.05 % bromphenol blue and 0.05% xylene cyanol was added prior to loading on a 10% sequencing gel.

Comparative Reactivities of OP-Cu, DMS and DEP

Primer-extension assays were used to compare the specificities of the different modification reagents. For reaction with OP-Cu, 0.1 pmol of RNA was preincubated for 5 min at 37° in 25 μ l of buffer composed of 50 mM Tris-HCl, pH 7.5, 50 mM NH₄Cl, 10 mM magnesium acetate, 2 mM β -mercaptoethanol, 200 μ M GTP. The scission reaction was carried out with 167 μ M OP, 37.5 μ M CuSO₄. To start the reaction, 2.5 μ l of 3-mercaptopropionic acid (final concentration, 7 mM) were added, and the reaction was allowed to proceed for 1 min at 90° or 5 min at 37° as indicated. Unless otherwise indicated, H₂O₂ was generated endogenously from the 1,10-phenanthroline-copper catalyzed oxidation of thiol. Addition of 7 mM hydrogen peroxide enhance the rate of the scission reaction but did not alter the specificity. The reactions were terminated by addition of 2.5 μ l of quench solution (28 mM 2,9-dimethyl-1,10-phenanthroline), 15 μ l stop solution (1.8 M sodium acetate, pH 5.0, 3 μ g *E. coli* tRNA, 4 mM mercaptoethanol, 40 mM EDTA) and 45 μ l of water saturated, redistilled phenol. For reactions involving dimethyl sulfate (DMS) or diethylpyrocarbonate (DEPC) (11), the buffer contained 1 mM DTT instead of mercaptoethanol. The reactions were initiated with the addition of 5 μ l of 120 mM DMS or 5 μ l of 20% DEPC (freshly prepared from refrigerated stock bottles) and incubated as described for the OP-Cu reaction. DEPC reactions, in which cacodylate replaced the Tris in the pH 7.5 buffer, were also carried out with essentially the same results. DMS and DEPC reactions were terminated by adding 15 μ l stop solution and 45 μ l phenol. After termination, all samples were sequentially extracted with phenol, phenol/chloroform and ether, and then precipitated with ethanol (12).

Primer Extension Assays

The recovered RNA was resuspended in 5 μ l reverse transcriptase buffer (50 mM Tris-HCl, pH 8.5, 60 mM NaCl, 10 mM DTT) and hybridized with 1 pmole of primer that had been 5'-end labeled by treatment with polynucleotide kinase and [γ - 32 P]ATP at ca. 5 μ Ci/pmol (13). The primer was a 17-mer complementary to the sequence beginning at residue 123 of the RNA transcript (residue numbered +78 in Table 1); this site corresponds to a pGEM2 vector sequence that is situated immediately distal to the inserted lac fragment. Primer extension was carried out with 2 units of AMV reverse transcriptase (Biorad), 300 μ M each deoxynucleoside triphosphate (in reverse transcriptase buffer) and additional magnesium acetate to give a final concentration of 6 mM, as described by Inoue and Cech (13). After completion of the incubations, the samples were mixed with dye-loading solution, boiled and loaded onto 8% (19:1 acrylamide:bisacrylamide) sequencing gels (20 x 40 x 0.02 cm) containing 7.0 M urea. Electrophoresis was carried out at 2000 V until the bromophenol blue migrated to the bottom of the gel. Autoradiographs of acid-fixed, dried gels were prepared on Kodak X-AR5 film with an intensifying screen. In primer extension assays, reactions were calibrated using dideoxysequencing ladders. Ribonuclease T₁ digests of terminally labeled RNA were used to calibrate the sequence gels used with this substrate.

Preparation of 5' and 3'-Labeled tRNA^{phe}

Yeast tRNA^{phe} was dephosphorylated with calf thymus intestinal alkaline phosphatase and 5' labeled with [γ - 32 P]ATP and bacteriophage T4 kinase (12). The labeled product was purified using a 12% polyacrylamide gel (1:19 crosslinked) in 8.3 M urea. 3' Labeling of tRNA^{phe} was carried out as described above for lac mRNA; it was purified in the same manner as the 5'-labeled tRNA.

OP-Cu Cleavage of Labeled tRNA

Labeled tRNA^{phe} in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl and 2mM MgCl₂ was cleaved with OP-Cu (167 μ M/38 μ M) in the presence of 7 mM H₂O₂ and 7 mM mercaptopropionic acid. The total volume was 10 μ l. After 5 min at 37°, the reaction was quenched with 2,9-dimethyl-OP. An equal volume of loading buffer consisting of 0.1% bromophenol blue, 0.1% xylene cyanol, 10 M urea and 20 mM EDTA was then added to the sample, which was subsequently loaded onto a 10% polyacrylamide/8.3 M urea gel for analysis.

Table 1
Sequence of RNA used in Comparing Reactivity of OP-Cu, DMS and DEPC.

	+1
5'pppgggagaccaagcugggcugcagguCGGCUCGUAUAAUGUGUGG	AAUUG
UGAGCGGAUAACAAUUUCACACAGGAAACAGCUAUGACCAUGAUUACG	
GAUUCACUGGaauccguguauuc <u>uauagugucaccuaaa</u>	--pGEM2

Capital letters: lac sequence; lower case letters: vector sequence; underlined sequence is hybridization site of the primer. +1 represents the start of lac transcription.

Materials

Materials were obtained from the following manufacturers: 1,10-phenanthroline (G.F. Smith); 3-mercaptopropionic acid, diethylpyrocarbonate, dimethylsulfate (Aldrich); cupric sulfate (Mallinkrodt); yeast tRNA^{phe} (Boehringer).

RESULTS

Two methods have been used to assay the reaction of the chemical reagents employed in this study. The most general method utilizes primer extension by reverse transcription (13). This assay detects modified bases which block the enzymatic synthesis of complementary DNA as well as sites where there is cleavage of the phosphodiester backbone. The second utilizes terminally ³²P-labeled RNA substrates and measures the products on a sequencing gel following modification (14,15). In this case, modification must lead to strand scission for the reaction to be detected. Modifications of the purines and pyrimidines by dimethyl sulfate (DMS) and diethylpyrocarbonate (DEPC) are not detected unless accompanied by subsequent mild base treatment (13,14).

OP-Cu reactivity was compared to the single-strand specific reagents, DMS and DEPC, using a fragment of the lac operon as substrate. By using a 17-base deoxyoligonucleotide primer that annealed just downstream of the lacZ sequence, primer extension assays detected reactions in the 5' region of this sequence. The transcript, which is 554 bases long, includes a portion of the lac UV-5 operator-promoter and lacZ gene from nucleotide positions -19 to +63, where +1 is the primary

Figure 1. Reaction of OP-Cu with lac mRNA. Lane 1: Control, lac mRNA treated with quench solution; lane 2: complete reaction; lane 3: reaction mixture lacking mercaptopropionic acid; lane 4: mixture lacking CuSO₄. Additional lanes show the RNA sequence obtained by dideoxy sequencing. Because primer extension detects the last intact nucleotide remaining on the RNA template, bands appearing in experimental lanes represent OP-Cu reactions occurring at the next residue upward on the sequence ladder. Numbers refer to positions of the RNA within the lac operon: transcription start site indicated as +1; arrow indicates the 5' end of the substrate RNA.

start site of lac transcription in vivo. (See Table 1 for sequence.) Reactions with the three reagents (DMS, DEPC and OP-Cu) were carried out under identical ionic conditions. As observed for the action of OP-Cu on DNA, the reaction of OP-Cu with RNA is dependent on added copper ion and thiol (Figure 1). The addition of exogenous hydrogen peroxide

Figure 2. Reaction of *lac* mRNA with OP-Cu, DMS and DEPC. Reactions run at 37° and 90° as indicated. Details as in Figure 1.

enhances the rate of the reaction without altering the specificity (data not shown).

All reactions were run at two temperatures, 37° and 90°. At the higher temperature, duplex structures may persist but the fraction of nucleotides in single-stranded regions should increase. The results shown in Figure 2 indicate that the sequence domains at 37° that are reactive to the chemical probes, DMS and DEPC, are also reactive to OP-Cu. Based on the generally accepted single-strand specificity of DEPC and DMS, these results indicate that OP-Cu also is selective for single-stranded regions.

The probable secondary structure of the *lac* transcript is presented

Figure 3. Sequence and secondary structure of substrate RNA. Computer folded structure shows the first 118 residues (those detected in the primer extension assay of the 554 nucleotide transcript). The sequence has been numbered relative to the start site of *lac* operon transcription in vivo indicated as +1.

in Figure 3; it was determined by a computer program written by Zuker and Steigler (16). It is supported by the pattern of chemical modification obtained using DMS and DEPC. Differences in the sites of reaction in single-stranded regions with the three reagents can be explained in the context of their specificity (13-15). When primer extension assays are used, DMS detects A and C in single-stranded regions whereas DEPC detects A, G and, to a lesser extent, C in these domains. Methylation of G at N-7 is not readily detected by primer extension unless strand scission is induced by mild base treatment. In contrast, since OP-Cu attacks the ribose moiety, it does not show specificity for any aspect of the base structure. As a result, in reactive regions there is a relatively even ladder of clustered products. At 90°, the reactivity of all three reagents is enhanced. The results with all three reagents suggest residual secondary structure persists in RNAs even at these elevated temperatures.

Direct Assay of Scission

Bands in primer extension assays can be due to modified bases which block reverse transcriptase or to scission of the phosphodiester bond. To demonstrate that the bands observed in Figures 1 and 2 with OP-Cu are due to strand scission and not base modification, RNAs labeled at either the 5' or 3' end were used as substrates and the reaction

Figure 4. Scission of *lac* mRNA by OP-Cu. A 3'-labeled 108 base transcript was treated with OP-Cu and the products analyzed on a sequencing gel. In addition to samples digested with OP-Cu, adjacent lanes show an undigested sample, and samples digested with RNase T₁ and NaOH. There are two species in this 3'-labeled RNA which differ in length by 1 base. The different species are indicated with a small circle or triangle.

products analyzed directly on sequencing gels. In these experiments, the reaction products were not treated with weak bases such as aniline or sodium borohydride, which are required to induce cleavage of DMS- or DEPC-modified RNA (14,15). The results shown in Figure 4 for the 3'-

Figure 5. Densitometer trace of OP-Cu scission pattern of tRNA analyzed on a sequencing gel. Regions of secondary structure are indicated. A. 5'-labeled tRNA^{Phe} B. 3'-labeled tRNA^{Phe}

labeled RNA indicate that strand scission is intrinsic to the nuclease activity of OP-Cu and proceeds spontaneously. In addition to underscoring the preference of OP-Cu for single-stranded regions, the

congruence of the results using two different methods of assay implies that the ribose moiety is the sole site of attack of OP-Cu with RNA. If there is any base reactivity with OP-Cu, it must lead directly to strand scission.

Secondary Structure Specificity in the Scission of tRNA by OP-Cu

The secondary structure specificity of OP-Cu was also examined using yeast tRNAP^{he} as substrate. The structure of this RNA has been determined by X-ray crystallography (17-19) and permits a further test for the single-strand preference implied by the above results. Both 5'- and 3'- labeled tRNAP^{he} were used to facilitate the examination of the digestion pattern of the entire sequence. The densitometric traces of the sequencing gel are presented in Figure 5. With 5'-labeled RNA, major regions of reactivity readily identified are the D loop (positions 13-20) and the anticodon loop (positions 32-38). In contrast, the residues at positions 10-12 and 23-25, which compose the double-helical stem of the D loop, are less reactive. The partially saturated dihydrouridine residues in the D loop are cleaved as readily as the C, U, G, and A residues in single-stranded regions.

With tRNAP^{he} labeled at the 3' end with ³²P-pCp, the digestion pattern from positions 34 to 72 can be examined. The results indicate that single-strand preference of OP-Cu provides a satisfactory first approximation for characterization of the range of the reagent's specificity. Low reactivity is observed in the double-stranded anticodon stem but also in the single-stranded, variable loop. On the other hand, position 50 within the T-stem region is as reactive as the G residue in the single-stranded T-loop. Unlike the D- and anticodon-loops, which demonstrate several reactive residues within the single-stranded region, this is the only reactive residue in the T-loop. The four most reactive residues observed with the 3'-labeled RNAs are positions 67-70 within the double-stranded, acceptor stem.

DISCUSSION

Two components of the chemical nuclease activity of 1,10-phenanthroline-copper are the reversible binding of the coordination complex to the nucleic acid and the efficiency of the oxidative scission chemistry:

Figure 6. Comparison of digestion patterns of tRNA^{Phe} by MPE and OP-Cu presented in cloverleaf (Figure 6A) and 3-D model (Figure 6B). Data for MPE are derived from references 23 and 24. Data from Fig. 5 were used in designating reactive sites. OP-Cu cleavage sites ----->, MPE cleavage sites (*). 3-D model is redrawn from reference 19.

Both steps influence the rate of the reaction. Since the oxidative species generated by the one-electron oxidation of the cuprous complex is not readily diffusible (1), no productive reaction with the nucleic acid is observed unless the coordination complex binds adjacent to its target. These aspects of the reaction are important for interpreting the specificity of OP-Cu in the scission of RNA.

The reaction pattern observed with the *lac* mRNA suggested stringent specificity of OP-Cu for single-stranded regions. This preference of OP-Cu for loop regions of RNA relative to the stem structures contrasts with the favored cutting within the B-helix of the stem of a synthetic DNA stem-loop. Only a single nucleotide within the DNA loop was attacked and it was adjacent to the helix (8). An important difference between these stem-loop structures is that the stem of RNA is an A-helix whereas that of the synthetic DNA is a B-helix. Since B-DNA is a more efficient substrate than A-DNA, part of the

preference for the loop structure of the RNA can be attributed to the decreased reactivity of the chemical nuclease for the A-structure (3).

The reaction with tRNAPhe, while supporting the tendency of OP-Cu to attack single-stranded regions, indicates that this specificity is not as rigid as the results with the lac mRNA imply. Scission in single-stranded regions is observed, but not all single-strand regions are nicked nor are all reactive sites in single-stranded regions. For example, several strong cutting sites are observed in the D- and anticodon- loops but only a single scission site is observed in the T-loop (G-57). In contrast, positions 67-70 which are in double stranded regions are also efficiently cut.

The reactivity of OP-Cu for tRNAPhe is summarized in Figure 6 using the cloverleaf and 3-D representations of the molecular structure (17-19). The 3-D representation of the cutting sites reveals three distinct regions of cutting. One region is the anticodon loop and includes sequence positions 32-38. The second is the juncture of the T- and D-loops and includes positions 13-20 of the T-loop, 57 of the D-loop and position 50 in the T-stem. The remaining prominent scission sites, 2, 5-8 and 67-70 are vicinal to the folded joint of the L-region. Because of the chemical properties of the scission activity, the cut nucleotides must be adjacent to a preferred binding site(s) for the tetrahedral coordination complex $(OP)_2Cu^+$. Generally, single stranded regions will provide binding sites for the coordination complex but specific tertiary folds of RNAs such as the joint of the L-region may generate strong binding sites for the coordination complex as well.

Methium-propyl-EDTA iron (MPE; 20,21) is also a chemical nuclease which reacts with RNA and DNA by an oxidative mechanism following an initial binding reaction (21,22). It is a strict structural analog of ethidium bromide and a more effective intercalating agent than $(OP)_2Cu^+$ (22). Unlike $(OP)_2Cu^+$, the majority of MPE reactive sites are therefore in double-stranded regions of tRNA (23,24) (See Figure 6 for comparison of reactive sites.) Detailed investigation of the reactivity of MPE with a ribosomal RNA fragment has underscored this reagent's specificity for the helical region of stem-loop structures (25). The different reaction of these reagents with RNA is mirrored by their reactivity with DNA. MPE reacts from an intercalative site and cleaves DNA with less dependence on sequence than OP-Cu which reacts from a site in the minor groove. Since the minor groove of the A-helix of an

RNA stem-loop does not provide a stable binding site for the tetrahedral $(OP)_2Cu^+$, the stranded bias of this reagent may result from the formation of molecular complexes, possibly of a charge-transfer with unstacked bases in single-stranded regions. Stable helical structures need not be disrupted for this type of complexation to occur. Weak molecular complexes could have multiple orientations leading to several cuts from a given binding site.

In summary, OP-Cu provides an approach for monitoring the secondary structure of RNAs especially when used in conjunction with other reagents. Generally, single-stranded regions are preferred sites of reaction relative to the A-helix of the stem of stem-loop structures. Nevertheless, specific tertiary interactions, especially in small, compact, stable RNAs may influence the binding of the coordination complex and lead to exceptions of this generalization. Despite this difficulty, the reagent is proving useful as a footprinting reagent to study protein-RNA interactions because it cuts at all nucleotides (26).

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