A transcription inhibitor specific for unwound DNA in RNA polymerase–promoter open complexes

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ABSTRACT The kinetically competent open complexes formed at prokaryotic and eukaryotic transcription start sites are efficiently nicked by the chemical nuclease activity of the 2:1 1,10-phenanthroline-copper(I) complex [(OP)2Cu+] and hydrogen peroxide. This reaction specificity has been attributed to the creation of a binding site(s) for redox-active tetrahedral (OP)2Cu+ when RNA polymerases form productive complexes with promoters. This proposal has been confirmed for the Escherichia coli lac UV-5 promoter by the demonstration that the 2:1 2,9-dimethyl-1,10-phenanthroline-copper(I) complex [(Me2OP)2Cu+], a redox-inactive isostere of (OP)2Cu+, protects the transcription start site from scission by the chemical nuclease activity. (Me2OP)2Cu+ is also an effective inhibitor of transcription. The inhibition of transcription and the protection from scission of the open complex by (OP)2Cu+ exhibit the same dependence on the concentration of (Me2OP)2Cu+. This redox- and exchange-stable species is a previously undescribed transcription inhibitor that binds to a site generated by the interaction of RNA polymerase with the promoter. Unlike the intercalating agent proflavine, which is also an effective transcription inhibitor, it does not displace the enzyme from the promoter. The ability of (Me2OP)2Cu+ to inhibit transcription may be partially responsible for its potent cytotoxicity.

Transcription of B-DNA is a multistep reaction which includes the recognition of the promoter by RNA polymerase (and accessory proteins), transcription initiation, elongation, and termination (1, 2). To permit synthesis of a faithful copy of the transcribed RNA, the enzymatic machinery must be able to form enzyme intermediates with single-stranded DNA structures. The "open complex" formed during transcription initiation (Eq. 1) is one intermediate in which the formation of single-stranded DNA has been unambiguously demonstrated. It forms after RNA polymerase binds to the promoter and the RNA polymerase-DNA complex undergoes a series of unimolecular isomerizations (Eq. 1) (3–7).

\[ \text{E} + \text{P} \xrightleftharpoons[d_1]{k_1} \text{E-P}_1 \xrightleftharpoons[d_2]{k_2} \text{E-P}_2 \xrightleftharpoons[d_3]{k_3} \text{E-P}_0 \rightarrow \text{NTPs} \text{ RNA} \]  

Here E is the RNA polymerase enzyme and P, P6, P7, and P0 are the free, closed, intermediate, and open forms of the promoter. Strong evidence for the formation of a single-stranded open complex at kinetically competent start sites has been provided by base-specific modification reagents such as potassium permanganate, which reacts with thymidine residues in single-stranded DNA (8–11), and dimethyl sulfate, which reacts with cytosine residues in single-stranded DNA (3). The chemical nuclease activity of the 2:1 1,10-phenanthroline-copper(I) complex [(OP)2Cu+] also cuts single-stranded or "strained" DNA at transcription start sites of certain genes (12). For example, the template strands of the Escherichia coli lac wild-type, Ps, and UV-5 promoters within open complexes are nicked at positions 6 to 4 nucleotides upstream of the start of transcription (13). Enhanced scission on the non-template strand is not observed. The unexpected specificity for transcription start sites of the curpous complexes of OP and some of its derivatives is not shared by other nucleolytic activities such as those of SI nuclease, micrococcal nuclease, DNase, ferrous EDTA, and methidium-propyl-EDTA (14, 15). The hyperreactivity of the chemical nuclease is apparently not restricted to prokaryotic transcription start sites, since the kinetically competent open complex of the major late adenovirus promoter is exceptionally reactive to the coordination complex, with cleavage reported on both strands upstream of the site of transcription initiation (16).

The specificity of the nuclease activity of (OP)2Cu+ has been attributed to the site-specific binding of the chelate either as the tetrahedral hydrophobic cation or linked to a targeting ligand such as a protein or nucleic acid. The hyperreactive sites observed in the footprinting of the open complex by (OP)2Cu+ therefore should be due to the site-specific binding of the tetrahedral chelate to the open complex of lac UV-5 with E. coli RNA polymerase. If this inference is valid, then a coordination complex isosteric with (OP)2Cu+ such as the redox-inactive 2:1 2,9-dimethyl-1,10-phenanthroline-copper(I) complex [(Me2OP)2Cu+] (17, 18) should bind at the transcription start sites which exhibit hyperreactivity with (OP)2Cu+. The scheme below indicates possible reactions of the open complex.

\[ (OP)_2Cu^+ \xrightarrow{\text{E-P}_0} (OP)_2Cu^+ \xrightarrow{\text{H}_2\text{O}_2} \text{Scission products} \]
\[ (Me_2OP)_2Cu^+ \xrightarrow{\text{E-P}_0} (Me_2OP)_2Cu^+ \xrightarrow{\text{H}_2\text{O}_2} \text{No reaction} \]

In this communication, we demonstrate that (Me2OP)2Cu+ blocks scission by (OP)2Cu+ of the lac UV-5 E. coli RNA polymerase open complex and inhibits transcription as well. Our results are fully consistent with the hypothesis that high-affinity binding sites for tetrahedral coordination compounds at lac promoters are best achieved with the redox-inactive isostere of (OP)2Cu+. The redox-inactivity of this complex will allow the nuclease to act independently of transcription initiation. The redox-inactive nuclease will also bind to other promoters, such as the UV-5 site. We propose that the redox-inactive isostere of (OP)2Cu+ is a competitive inhibitor of transcription initiation, whereas the redox-active nuclease acts to promote transcriptional termination.

Abbreviations: OP, 1,10-phenanthroline (ortho-phenanthroline); Me2OP, 2,9-dimethyl-1,10-phenanthroline; Me2Ph2OP, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline; Ph2OP, 4,7-diphenyl-1,10-phenanthroline.

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plexes such as (OP)2Cu+ and (Me2OP)2Cu+ are generated at kinetically competent transcription start sites. The oxidatively stable (Me2OP)2Cu+ is therefore a transcription inhibitor which might find general use in the design of gene-specific inactivation reagents.

MATERIALS AND METHODS

Enzymes and Reagents. The sources of enzymes and reagents are as follows: E. coli RNA polymerase, Pharmacia; T4 polynucleotide kinase, EcoRI, and Pvu II, GIBCO/BRL; calf intestinal alkaline phosphatase, Boehringer Mannheim; nuclease triphosphates, Promega; OP, Me2OP, and 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (Me2Ph2OP), GFS Chemicals (Columbus, OH); copper(II) sulfate, Mallinkrodt; and proflavine, Sigma.

Isolation and Labeling of the lac UV-5 Promoter. lac UV-5 was cloned in pUC87, a plasmid constructed from pUC18 and pHh314 (19). The resulting plasmid, pBV87, was digested with EcoRI to generate a 203-bp fragment containing the lac UV-5 promoter. This fragment was then dephosphorylated with calf intestinal alkaline phosphatase, isolated by gel electrophoresis, and 5′-end labeled by using T4 polynucleotide kinase and [γ-32P]ATP. A Pvu II digestion was then performed to generate a singly labeled (on the template strand) 186-bp fragment, which was isolated by gel electrophoresis.

(PO)2Cu+ Footprinting of an Open Complex. Five microliters of 5′-32P-labeled lac UV-5 promoter was incubated with 5 μl of either 2× transcription buffer (80 mM Tris-HCl, pH 8/100 mM KCl/20 mM MgCl2) or RNA polymerase (400 nM) previously diluted into 2× transcription buffer for 10 min at 37°C. Two microliters of stock solutions containing (Me2OP)2Cu+, (OP)2Fe+2, (Me2Ph2OP)2Cu+, or proflavine was added, and the incubation was continued for an additional 2 min. To effect scission, OP, CuSO4, and 3-mercaptopropionic acid (MPA) were added to a final concentration of 60 μM OP, 30 μM Cu+2, and 6.9 mM MPA, allowing the in situ generation of (OP)2Cu+ and H2O2. Reactions were quenched by the addition of EDTA to a final concentration of 50 mM, and DNA was precipitated with ethanol. The pellet was washed with 70% ethanol, dried, and suspended in loading dye [0.1% bromophenol blue/0.1% xylene cyanol/1 mM EDTA/80% (vol/vol) deionized formamide]. Samples were heated at 95°C for 3 min and then electrophoresed on a denaturing gel [10% polyacrylamide/7 M urea/1× TBE (0.09 M Tris borate, pH 8.3/2 mM EDTA)].

In Vitro Transcription. Unlabeled 203-bp fragment (1.5 pmol) was incubated with RNA polymerase at a final concentration of 150 nM in a buffer containing 40 mM Tris-HCl at pH 8, 50 mM KCl, 10 mM MgCl2, and 33 mM dithiothreitol at 37°C for 10 min. Three microliters of water or a stock solution of the inhibitor was added, and the incubation was continued for an additional 2 min. One microliter of [α-32P]UTP (specific activity 400 Ci/mmol; 1 Ci = 37 GBq) and 3 μl of a nucleotide mixture (1.5 mM ATP/0.625 mM UTP/0.625 mM GTP/0.625 mM CTP) were added. After 30 min, the transcription was quenched by the addition of 2 μl of 0.5 M EDTA and 8 μl of loading dye. The mixture was heated at 95°C for 3 min and loaded on a denaturing 20% polyacrylamide gel.

Gel Retardation Assays. The promoter-polynucleosome complex was formed as described above. Two microliters of a solution containing either the chelate or proflavine was added and the incubation was continued at 37°C for 2 min. One-half microliter of heparin at 1 mg/ml was added and the reaction mixture was incubated for 1 min. Four microliters of Ficoll/0.1% bromophenol blue was added and the reaction products were loaded on a nondenaturing 5% polyacrylamide gel.

RESULTS

Site-Specific Binding of (Me2OP)2Cu+ to E. coli RNA Polymerase-lac UV-5 Open Complex. The nuclease activity of (OP)2Cu+ has been studied with the lac wild-type, PS, and UV-5 promoters both in the absence of proteins and in the presence of E. coli RNA polymerase and the catabolite activator protein, a positive transcription regulator (13, 20). (OP)2Cu+ reacts with the free DNA of the mutant promoters differently in the region of the Pribnow box. Localized alterations in the minor groove geometry which alter the binding of the tetrahedral (OP)2Cu+ are probably responsible for the sequence dependent variability of the reactivity (21, 22). (Me2OP)2Cu+ does not inhibit or alter the scission of the promoter DNAs by (OP)2Cu+ at concentrations as high as 100 μM (23).

Despite the difference in scission patterns of the free promoter DNAs, (OP)2Cu+ cleaves the open complexes formed by these three lac promoters with E. coli RNA polymerase equivalently (13). These results suggest that the open complexes formed with the different promoters are similar, if not identical, in structure. Given the apparent equivalence of the three open complexes, the scission pattern of the "open" lac UV-5 RNA polymerase complex has been used to monitor the binding of the (Me2OP)2Cu+. If the sites hyperreactive to (OP)2Cu+ in the open complexes are due to the generation of a specific binding site for the tetrahedral chelate, then (Me2OP)2Cu+ should also bind at these sites with high affinity and block the scission reaction. In Fig. 1, the protection by (Me2OP)2Cu+ of the cleavage of the open complex by (OP)2Cu+ is observed in the concentration range 50–180 μM. The concentration dependence of the protection of scission by (Me2OP)2Cu+ is cooperative, with a Hill coefficient of approximately 1.8.

Two features of the protection by (Me2OP)2Cu+ suggest that this coordination complex binds to the open complex and does not displace RNA polymerase from the promoter. The first is that (Me2OP)2Cu+ generates a new site of scission at −2. This is consistent with the binding of (Me2OP)2Cu+ to the

![Fig. 1. Protection of E. coli RNA polymerase lac UV-5 open complex by (Me2OP)2Cu+ from scission by (OP)2Cu+. Lanes 1 and 3, 5′-32P-labeled lac UV-5 promoter cut with (OP)2Cu+ in the absence (lane 1) or presence (lane 3) of RNA polymerase; lane 2, Maxam-Gilbert G+A sequencing lane; lanes 4–7, open complex incubated with (Me2OP)2Cu+ at 180 μM (lane 4), 60 μM (lane 5), 20 μM (lane 6), or 7 μM (lane 7) prior to cutting by (OP)2Cu+ at 30 μM for 4 min at 37°C.](image-url)
melted complex but not by the displacement of the enzyme from the promoter. Second, addition of (Me2OP)2Cu+ to the open complex does not restore the strong scission sites observed with the free lac UV-5 DNA in the region of the Prünew box (20).

Inhibition of Enzyme Activity. The protection from the scission reaction suggests that (Me2OP)2Cu+ should inhibit RNA synthesis from the lac UV-5 promoter. The assay of (Me2OP)2Cu+ as an inhibitor of transcription was carried out by incubating various concentrations of the coordination complex with E. coli RNA polymerase and lac UV-5 and then adding a mixture of ribonucleoside triphosphates to initiate RNA synthesis. Transcription inhibition and the protection of lac UV-5 from scission in the open complex show an identical dependence on concentration of (Me2OP)2Cu+ (Fig. 2). Therefore both processes can be attributed to the binding of (Me2OP)2Cu+ to the open complex. Double-reciprocal plots indicate that (Me2OP)2Cu+ is a partially competitive inhibitor with respect to ATP or ApA.

The dissociation of lac UV-5 DNA from RNA polymerase by (Me2OP)2Cu+ is an alternative mechanism to account for the protection of (OP)2Cu+ scission of the open complex and the inhibition of enzyme activity. Although the digestion patterns presented in Fig. 1 support the formation of a ternary complex composed of chelate, enzyme, and DNA, gel retardation assays were used as an independent method to monitor the stability of RNA polymerase-promoter complexes in the presence of (Me2OP)2Cu+. The open complex was incubated with increasing concentrations of the coordination complex both in the presence and in the absence of ApA and UTP, which would permit the synthesis of the tetranucleotide AAUUA. At concentrations where extensive inhibition of both the cleavage reaction and enzymatic activity is observed, the enzyme is not displaced from the promoter as measured by gel retardation (Fig. 3).

In contrast, proflavine is an intercalating agent which inhibits transcription from the lac UV-5 promoter at concentrations as low as 0.01 mM. When it is added to a solution of lac UV-5 and RNA polymerase, the scission sites characteristic of the open complex are not observed (Fig. 4A). Moreover, the strong scission of the free DNA by (OP)2Cu+ at sequence positions −13 to −10 reappears. These results, which suggest that proflavine inhibits transcription by causing the dissociation of RNA polymerase from the promoter, are supported by gel retardation analyses demonstrating the displacement of the enzyme from the DNA in the relevant concentration range (Fig. 4B, lanes 3–5).

Specificity of Inhibitor Binding Site of the Open Complex.

Inhibition of the binding site generated in the open complex for (OP)2Cu+ and (Me2OP)2Cu+ has a definite specificity for tetrahedral coordination complexes. For example, the octahedral (OP)2Fe2+ complex neither blocks the scission reaction nor inhibits transcription. However, not all tetrahedral complexes bind to the open complex. The cuprous complexes of the redox-inactive 2-carboxy-1,10-phenanthroline and 2-hydroxymethyl-1,10-phenanthroline do not inhibit scission or transcription.

The cuprous complexes of 4,7-diphenyl-1,10-phenanthroline (Ph2OP) and Me2Ph2OP allow comparison of the binding and scission properties of another pair of isosteres. The cuprous complex of Ph2OP is a redox-active coordination complex which cleaves free DNA in the presence of hydrogen peroxide in a sequence-independent reaction (24). Unlike the pattern seen with the cuprous complexes of OP and its 5-substituted derivatives, there are no prominent hypersensitive sites in the scission of free DNA. This complex fails to cleave the single-stranded DNA in the RNA polymerase-lac UV-5 open complex. Its redox-inactive isostere, the (Me2Ph2OP)2Cu+ complex, neither inhibits transcription nor blocks scission of the open complex by (OP)2Cu+.

FIG. 3. Gel retardation of open and initiation complexes in the presence of (Me2OP)2Cu+. Lane 1, 5'-32P-labeled lac UV-5 DNA; lane 2, 5'-32P-labeled lac UV-5 incubated with RNA polymerase; lanes 3–5, open complex incubated with 22 μM (lane 3), 67 μM (lane 4), or 200 μM (lane 5) (Me2OP)2Cu+; lane 6, open complex incubated with ApA and UTP; lanes 7–9, open complex incubated with ApA, UTP, and 22 μM (lane 7), 67 μM (lane 8), or 200 μM (lane 9) (Me2OP)2Cu+.

DISCUSSION

The specific cleavage of the template strand of lac UV-5 by (OP)2Cu+ suggested that the formation of a transcriptionally competent open complex with E. coli RNA polymerase generates a binding site(s) for the tetrahedral chelate. This hypothesis has been supported by the demonstration that the redox-inactive isostere of (OP)2Cu+, (Me2OP)2Cu+, blocks the cleavage reaction and is an effective transcription inhibitor which binds specifically and tightly to the open complex formed by E. coli RNA polymerase and the lac UV-5 promoter. (Me2OP)2Cu+ represents a previously undescribed type of transcription inhibitor that binds to a new site generated at the catalytic center of E. coli RNA polymerase.

Three experiments demonstrate that this tetrahedral coordination complex binds to a site generated by the melting of double-stranded DNA by RNA polymerase. First, (Me2OP)2Cu+ blocks the nicking of the template strand at positions 6 to 4 nucleotides upstream of the start of tran-
The inhibition of both transcription and scission of the open complex by (Me2OP)2Cu+ has a Hill coefficient of 1.8, suggesting that more than one binding site is generated for the chelate. The new scission site at position −2 in the presence of (Me2OP)2Cu+ provides direct support for this conclusion because it suggests that this complex and (OP)2Cu+ can bind to the open complex simultaneously. The existence of more than one binding site may also explain why multiple sites of scission are observed in the reaction of the various OP-Cu+ chelates with the open complex. The specificity of these sites for other tetrahedral cations should be probed further. Presently it is known that substitution at the 4 and 7 positions of OP with phenyl groups abolishes binding. Since the cuprous complexes of 2-carboxy- and 2-hydroxyxymethyl-1,10-phenanthroline are not effective inhibitors, a strong preference for methyl groups at the 2 and 9 positions is indicated. The strong preference for a tetrahedral geometry is reflected by the lack of affinity of the octahedral (OP)2Fe2+ complex for the open complex.

The discovery that (Me2OP)2Cu+ is an effective inhibitor of transcription provides one possible explanation for the previously puzzling biological effects of this redox- and exchange-stable coordination complex. Unlike (OP)2Cu+, (Me2OP)2Cu+ is not mutagenic in the Ames test because it does not have an efficient nuclease activity (25). However, it is toxic to a variety of prokaryotic and eukaryotic cells (25–30). For example, (Me2OP)2Cu+ kills L1210 cells in culture at concentrations as low as 0.5 μM. Cuprous ion is essential for the cytotoxicity of Me2OP and cannot be replaced by iron. However, transcription inhibition may not be the sole mechanism for the toxicity of (Me2OP)2Cu+. Since (OP)2Cu+ exhibits hyperreactivity at replication origins in yeast (30), (Me2OP)2Cu+ may also bind to the melted DNA regions which might be formed at the initiation site of DNA replication. In either case, the stability of (Me2OP)2Cu+ would contribute to its cytotoxicity because a high concentration will persist intracellularly.

In summary, simple coordination complexes of OP have shown a remarkable range of selective interactions with RNA and DNA (31–35). This repertoire is extended by the discovery that (Me2OP)2Cu+ has affinity for the melted DNA structures formed at the active site of E. coli RNA polymerase with the strong lac UV-5 promoter. Preliminary work indicates that this coordination complex is an effective inhibitor of other transcription units. (Me2OP)2Cu+, and molecules structurally related to it, may form a valuable family of transcription inhibitors that will be useful as probes of enzyme mechanism as well as selective pharmacological agents.

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