INHIBITION OF E. coli DNA POLYMERASE I BY 1,10-PHENANTHROLINE

Vito D'Aurora, Andrew M. Stern, and David S. Sigman

Department of Biological Chemistry, School of Medicine
and Molecular Biology Institute, UCLA
Los Angeles, California 90024

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Summary. A 1,10-phenanthroline-cuprous ion complex is a potent reversible inhibitor of E. coli DNA polymerase I yielding 50% inhibition in the micromolar concentration range. The 2:1 1,10-phenanthroline-cuprous ion complex is most probably the inhibitory species. Complexes of cupric ion and 1,10-phenanthroline have no apparent kinetic effect. The previously reported inhibition of the enzyme by 1,10-phenanthroline (1,2) is most likely due to the formation of this complex from thiols normally added to the assay mixtures and trace amounts of cupric ion invariably present notwithstanding reasonable precaution. The reversible and instantaneous 1,10-phenanthroline inhibition observed for other polymerases may be due to this unique inhibitory species and not coordination of a catalytically important zinc ion at the active site by the chelating agent.

The inhibition of enzymatic activity by the chelating agent, 1,10-phenanthroline (OP) often implies the presence of a transition metal ion, frequently zinc ion, at the active site. The sensitivity of several DNA and RNA polymerases to OP has prompted a successful search for this metal ion in a number of enzymes including E. coli DNA polymerase I (1,2), DNA dependent RNA polymerase from E. coli (3), and the RNA dependent DNA polymerase from Avian Myeloblastosis virus (4), as well as others.

The reported reversible inhibition of E. coli DNA polymerase I (Pol I) has been assumed to result from the formation of an enzyme-1,10-phenanthroline complex in which the main locus of attachment of the chelating agent is the tightly bound zinc ion (1). In attempting to determine the catalytic function of the zinc ion in Pol I, we discovered that the reversible inhibition of Pol I by 1,10-phenanthroline is not observed unless exogenous thiols are present in the assay even though sulfhydryl compounds have an insignificant effect on the catalytic activity in the absence of chelating agent. The explanation for this unusual observation is that 1,10-phenanthroline inhibits

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Pol I by forming a complex with cuprous ion which is inhibitory in the micromolar concentration range. A thiol is required to reduce trace amounts of cupric ion, present as a contaminant in the assay mixture, to cuprous ion.

MATERIALS AND METHODS

Enzyme. E. coli DNA polymerase I was prepared from E. coli strain B according to Jovin et al. (5). Fraction VII was routinuely used in kinetic studies but comparable results were also obtained with Fraction IV.

Substrates. The sodium salts of dATP, TTP (Sigma), ^{3}H-dATP and ^{3}H-TTP (New England Nuclear), β-mercaptoethanol (MCB), dithiothreitol (Aldrich), 1,10-phenanthroline (Eastman and Aldrich), 2-propanethiol (Aldrich) and 3-mercaptopropionic acid (Aldrich) were purchased in the highest grade commercially available. Poly d(A-T), the primer-template most generally used, was purchased from Miles Laboratories.

Assay. The poly d(A-T) directed incorporation of either ^{3}H-dATP or ^{3}H-TTP into an acid insoluble form catalyzed by Pol I was measured by a published procedure using enzyme in the nanomolar concentration range (6).

RESULTS

Initial measurements of the inhibition of E. coli DNA polymerase I by 1,10-phenanthroline in the presence of mercaptoethanol or dithiothreitol revealed that the extent of inhibition of the enzyme at fixed concentrations of chelating agent (2×10^{-4}M) was dramatically dependent on thiol concentration (Fig. 1). No inhibition was observed in the absence of mercaptan even though neither thiol at the concentrations indicated had any effect on the enzyme activity in the absence of 1,10-phenanthroline. Coordination was an essential component in the 1,10-phenanthroline inhibition since non-chelating analogs such as 9-phenanthroic acid and 4,7-phenanthroline did not affect the rate under comparable conditions. 5-Methyl and 5-chloro-1,10-phenanthroline also required the presence of thiols such as mercaptoethanol to potentiate the inhibition. Higher mercaptoethanol concentrations were required to relieve the inhibition by the more effective chelating agents which decrease in coordinating ability in the order 5-methyl-1,10-phenanthroline, 1,10-phenanthroline, and 5-chloro-1,10-phenanthroline (7). Thiols such as propanethiol and 3-mercaptopropionic acid, which are not effective chelating

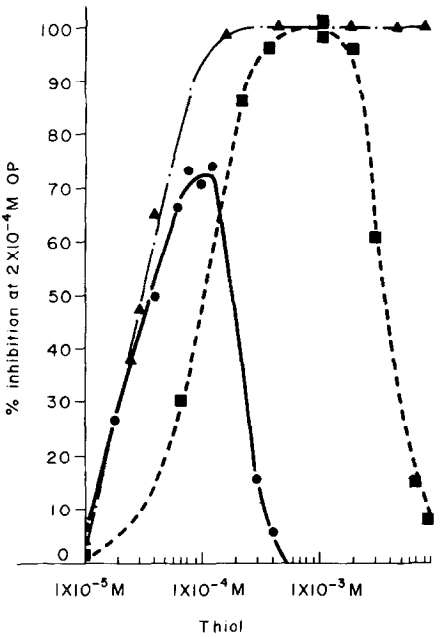

Fig. 1. Percent inhibition at 2 x 10^{-4}M orthophenanthroline (OP) as a function of dithiothreitol (——), 2-mercaptoethanol (---), and 3-mercaptopropionic acid (-·-) concentration at 37°. The 0.3 ml assay mixture consisted of .067 M Tris-HCL buffer, 3.3 x 10^{-5} M dATP, 3.3 x 10^{-5} M TTP, 6.7 x 10^{-3} M $MgCl_2$, and 8 µg/ml poly d(A-T) where dATP was radioactively labeled with ^3H. Assays were initiated by the addition of enzyme to an assay mixture already containing the concentration of thiol represented in the figure.

agents, potentiate the inhibition without relieving it at elevated concentrations (Fig. 1).

Our observations are difficult to rationalize in terms of the chemistry of the one free sulfhydryl and one disulfide bond present in the enzyme (6) or in terms of the zinc ion. The free sulfhydryl is probably not responsible for these effects since the enzyme can be modified with mercuric nitrate without any loss of activity (8). Attempts to trap any product resulting from the reduction of the lone disulfide by various mercaptans with N-ethyl maleimide

TABLE 1

Inhibition of Pol I by Cuprous Complexes of 1,10-Phenanthroline

Additions to Assay Mixtures*

Exp.	Cu^{+2} (M)	OP (M)	MPA^{+} (M)	% control
1	0	0	5×10^{-4}	103
2	1×10^{-5}	0	0	101
3	1×10^{-5}	0	5×10^{-4}	95
4	0	2×10^{-5}	0	105
5	0	2×10^{-5}	5×10^{-4}	42
6	1×10^{-5}	2×10^{-5}	0	109
7	1×10^{-5}	2×10^{-5}	5×10^{-4}	0
8	0	5×10^{-6}	5×10^{-4}	95
9	5×10^{-7}	5×10^{-6}	5×10^{-4}	65
10	1×10^{-6}	5×10^{-6}	5×10^{-4}	13
11	2×10^{-6}	5×10^{-6}	5×10^{-4}	5
12	5×10^{-6}	10^{-4}	5×10^{-4}	0
13	10^{-4}	5×10^{-6}	5×10^{-4}	66

* Assay mixture contained 67mM potassium phosphate buffer, pH 7.0; 6.7mM $MgCl_2$; 5×10^{-5}M ^{3}H-dATP (specific activity 10^5 dpm/nmole); 5×10^{-5}M TTP, .56 µg/ml poly d(A-T). Assay (T = 37°) initiated by addition of enzyme (1nM). MPA is the last component added prior to polymerase.

+ MPA abbreviation for 3-mercaptopropionic acid.

and Ellman's reagent were unsuccessful. The presence of mercaptan in the assay mixture was essential for 1,10-phenanthroline inhibition independent of the prior treatment of the enzyme with reducing agent.

An explanation for these unusual observations became apparent when the experiments summarized in Table 1 were performed. The potent inhibition observed in the presence of 1,10-phenanthroline, cupric ion, and 3-mercaptopropionic acid strongly indicates that a 1,10-phenanthroline-cuprous ion complex is a very effective inhibitor of the poly d(A-T) directed synthesis of DNA by Pol I. Our results clearly show that the cupric complex with 1,10-phenanthroline is not an effective inhibitor (expt. 6, Table 1). Cobalt(ous), zinc, ferric, ferrous and cadmium ions failed to inhibit under comparable conditions.

These observations strongly imply that the inhibition observed in the absence of added cupric ion is due to the presence of this metal ion as a contaminant in our standard assay mixture. Analysis for trace metals in our assay mixture by optical emission spectroscopy confirmed that cupric ion was present at concentrations of 5×10^{-7}M in our assay mixtures. Major sources of cupric ion which were identified were the phosphate buffer and the magnesium chloride even though reagent grade chemicals were always used. Extraction of assay mixtures with dithizone markedly diminished the extent of 1,10-phenanthroline inhibition in the presence of mercaptan, further emphasizing the importance of trace metals for the observed kinetic effects. Even the abolition of 1,10-phenanthroline inhibition by mercaptoethanol and dithiothreitol is explicable in terms of a 1,10-phenanthroline-cuprous ion complex being the inhibitor. At high concentrations, these mercaptans, which are also chelating agents, successfully compete with the 1,10-phenanthroline for the cuprous ion and reduce the concentration of the kinetically important species.

DISCUSSION

The most apparent conclusion from these studies is that the mechanism of the reversible 1,10-phenanthroline inhibition of *E. coli* DNA polymerase I is not its coordination to zinc ion at the active site as has been previously proposed (1,2). Zinc ion may be playing a central catalytic role at the active site of Pol I but inhibition by 1,10-phenanthroline in the assay mixture is not an appropriate criterion to infer it. Some essential role for zinc ion in Pol I nevertheless is still implied by the complete loss of activity accompanying its removal upon dialysis against 1,10-phenanthroline (2).

The precise mechanism by which the 2:1 1,10-phenanthroline-cuprous complex inhibits the polymerase is at present uncertain. We favor the hypothesis

that it acts as a reversible inhibitor because its three dimensional structure is fortuitously complementary to the active site of the enzyme. The reversible inhibition of beef heart mitochondrial ATPase by the 3:1 4,7-diphenyl 1,10-phenanthroline-ferrous complex provides a precedent for a phenanthroline-metal ion complex serving as a reversible inhibitor (9). We have found that this exchange inert complex is also a very effective inhibitor of Pol I yielding 50% inhibition at a concentration of 5×10^{-6}M using the assay conditions of Table 1.

Our studies strongly suggest that the inhibition by 1,10-phenanthroline reported for other polymerases should be re-examined in order to determine the generality of the phenomena reported here. Thiols are routinely used in the assay of polymerases and it is likely that the levels of cupric ion were similar to those observed by us. The demonstration of a similar mechanism of inhibition would prohibit any conclusion that 1,10-phenanthroline inhibition reflects a central role for the tightly bound zinc ion. It would, however, lead to the intriguing possibility that specific inhibitors of the various polymerases could be generated by systematic variation of inorganic ion and phenanthroline structure. These inhibitory complexes could then classify polymerases and possibly help in exploring the mechanism of DNA replication in complex systems where genetic approaches are ambiguous or impossible.

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