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1,10-Phenanthroline-Cuprous Ion Complex, a Potent Inhibitor of DNA and RNA Polymerases

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

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

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Summary. The inhibition by 1,10-phenanthroline of E. coli DNA polymerase I has recently been attributed to the formation in the assay mixtures of a unique and effective inhibitor, the 2:1 1,10-phenanthroline-cuprous ion complex (1). We have now found that this coordination complex is also an effective inhibitor of E. coli DNA dependent RNA polymerase, Micrococcus luteus DNA dependent DNA polymerase, and T-4 DNA dependent DNA polymerase. This conclusion is based either on the requirement of a thiol for 1,10-phenanthroline inhibition or on the reversal of 1,10-phenanthroline inhibition by the non-inhibitory cuprous ion specific chelating agent 2,9-dimethyl-1,10-phenanthroline. 2,2',2"-Terpyridine is also very effective at relieving 1,10-phenanthroline inhibition. The reversal of 1,10-phenanthroline inhibition should be attempted before it is claimed that 1,10-phenanthroline inhibits any polymerases by coordinating a zinc ion at the active site.

INTRODUCTION

DNA and RNA polymerases purified from a variety of sources contain tightly bound zinc ions (2-5). In many cases these enzymes are inhibited reversibly by the chelating agent 1,10-phenanthroline and it has been previously assumed that this inhibition reflects the coordination of a catalytically important zinc ion at the active site. However, recently we have reported that the reversible inhibition of E. coli DNA polymerase I by 1,10-phenanthroline is due to the formation of a 2:1 1,10-phenanthroline-cuprous ion complex which is inhibitory in the micromolar concentration range (1). This coordination complex forms in assay mixtures because trace amounts of cupric ion are present in reagent grade chemicals and sulfhydryl compounds are usually added to assay mixtures to enhance activity.

It was of obvious interest to determine if other polymerases reported to

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be sensitive to 1,10-phenanthroline are inhibited by the same mechanism as E. coli DNA polymerase I. In the present communication, we wish to report that E. coli RNA polymerase, T-4 DNA polymerase and M. luteus DNA polymerase are also effectively inhibited by the 1,10-phenanthroline-cuprous ion complex. This conclusion is based either on the necessity for thiols in the assay mixture for the expression of 1,10-phenanthroline inhibition or the relief of 1,10-phenanthroline inhibition by non-inhibitory chelating agents such as 2,2',2"-terpyridine and 2,9-dimethyl-1,10-phenanthroline. The latter chelating agent is specific for cuprous ion.

MATERIALS AND METHODS

Enzymes. E. coli DNA polymerase I was prepared to fraction VII according to Jovin et al. (6). M. luteus DNA polymerase and E. coli RNA polymerase were purchased from P-L Biochemicals. T-4 DNA polymerase, prepared according to the method of Goulian et al. (7) was a gift of Dr. Rolf Sternglanz.

Substrates. The sodium salts of dATP, TTP, ATP, UTP (Sigma), ^{3}H-dATP, ^{3}H-TTP and ^{3}H-ATP (New England Nuclear), 3-mercaptopropionic acid, 1,10-phenanthroline (Aldrich), all other chelating agents (G. F. Smith) and template/primers (Miles), were purchased in the highest grade commerically available.

Assay. The poly d(A-T) directed incorporation of ^{3}H-dATP into an acid insoluble form catalyzed by E. coli DNA polymerase I was measured by a published procedure using enzyme in the nanomolar concentration range (8).

A similar procedure was used for assaying the other polymerases. The substrates and template/primers for the enzymes were: 1) 0.2 mM ^{3}H-ATP, 0.2 mM UTP, 1 μgm/ml poly d(A-T) for RNA polymerase; 2) 0.05 mM ^{3}H-dATP, 0.05 mM TTP, 1 μgm/ml poly d(A-T) for Pol I of E. coli, M. luteus and T-4 DNA polymerases.

All assays were initiated by adding 0.05 ml (0.2 units) of diluted enzyme to 0.150 ml of assay mixture at 37° C prepared in 0.1 M Tris-HCl pH 7.0, .005 M $MgCl_2$, with or without 0.005 M 3-mercaptopropionic acid. In those assays containing 1,10-phenanthroline and the other cuprous ion chelating agent, 1,10-phenanthroline was the last component added before initiation of the reaction with enzyme.

RESULTS AND DISCUSSION

Results. The initial demonstration that the 2:1 1,10-phenanthroline-cuprous ion complex inhibited E. coli DNA polymerase I was largely based on the synergistic inhibition of the enzyme by cupric ion and 1,10-phenanthroline expressed only in the presence of thiols. The same series of experiments could not be readily performed on most polymerases because, unlike E. coli

TABLE 1

Thiol Dependence of 1,10-Phenanthroline Inhibition

ENZYME	OP[a] M	MPA[b] M	%‡
E. coli RNA Polymerase	10^{-4}	---	98
	10^{-4}	$5X10^{-3}$	0
M. luteus DNA Polymerase	10^{-4}	---	100
	10^{-4}	$5X10^{-3}$	0

See text for assay conditions. ‡% of control with no chelators.
[a]OP = 1,10-phenanthroline. [b]MPA = mercaptopropionic acid.

DNA polymerase I, they are strongly inhibited by either cuprous or cupric ion alone and thiols are sometimes absolutely required for catalytic activity. However, the data summarized in Table 1 indicate that it was possible to demonstrate a thiol dependence of the inhibition of E. coli DNA dependent RNA polymerase and M. luteus DNA dependent DNA polymerase since acceptable levels of activity exist in the absence of added thiols. In these experiments, the concentration of cupric ion present as a contaminant in magnesium chloride was there in sufficient concentrations to generate the inhibitory complex.

Since T-4 DNA dependent DNA polymerase requires thiol for stable levels of activity, it was necessary to devise a new criterion, other than thiol dependence for inhibition, to demonstrate that coordination complexes of 1,10-phenanthroline and not the chelating agent itself were responsible for the observed inhibition. The reversal of 1,10-phenanthroline inhibition by chelating agents which themselves were non-inhibitory proved to be a useful means to accomplish this goal. Inhibition would be relieved by these chelating agents since the concentration of the kinetically important 1,10-phenanthroline complex would be reduced. This approach is illustrated by the data summarized

TABLE 2

Reversal of 1,10-Phenanthroline Inhibition of *E. coli* DNA Polymerase I by Chelating Agents

OP[a]	NC[b]		Ter[c]		PA[d]	
(M)	(M)	%‡	(M)	%‡	(M)	%‡
0	10^{-5}	98	10^{-5}	99	10^{-5}	97
0	10^{-4}	100	10^{-4}	101	10^{-4}	96
0	10^{-3}	112	10^{-3}	98	10^{-3}	126
$5X10^{-5}$	0	0	0	0	0	0
$5X10^{-5}$	10^{-5}	4	10^{-5}	13	10^{-5}	0
$5X10^{-5}$	10^{-4}	97	10^{-4}	94	10^{-4}	0
$5X10^{-5}$	10^{-3}	107	10^{-3}	95	10^{-3}	0

See text for assay conditions. (Concentration of 1,10-phenanthroline in each assay is indicated in the first column.) ‡% of control with no chelators. [a]OP = 1,10-phenanthroline. [b]NC = 2,9-dimethyl-1,10-phenanthroline. [c]Ter = 2,2',2"-terpyridine. [d]PA = 9-phenanthroic acid.

in Table 2 which describes the reversal of the 1,10-phenanthroline inhibition of *E. coli* DNA polymerase I by the chelating agents 2,2',2"-terpyridine and 2,9-dimethyl-1,10-phenanthroline. These chelating agents do not have a kinetic effect in the absence of 1,10-phenanthroline. Since a non-chelating phenanthroline analog, 9-phenanthroic acid, does not relieve 1,10-phenanthroline inhibition, their chelating properties must be responsible for the observed relief of inhibition.

The reversal of inhibition by 2,9-dimethyl-1,10-phenanthroline, which is specific for cuprous ion (9), stresses the central role of copper ion in the inhibition. However, the thiol dependence of the inhibition is essential to

TABLE 3

Reversal of 1,10-Phenanthroline Inhibition of
E. coli RNA Polymerase by Chelating Agents

OP[a] (M)	NC[b] (M)	NC[b] %‡	Ter[c] (M)	Ter[c] %‡
0	---	---	10^{-5}	99
0	10^{-4}	110	10^{-4}	101
0	10^{-3}	104	10^{-3}	98
$5X10^{-5}$	0	0	0	0
$5X10^{-5}$	---	---	10^{-5}	100
$5X10^{-5}$	10^{-4}	98	10^{-4}	101
$5X10^{-5}$	10^{-3}	106	10^{-3}	81

Assay procedures described in text. Abbreviations and organization identical to Table 2.

conclude the importance of cuprous rather than cupric ion. Cuprous ion might be generated in the reducing environment of the assay mixture, in the presence of chelating agents specific for it. 2,2',2"-Terpyridine is an effective but non-specific chelating agent and is a good ligand to test for the possible importance of 1,10-phenanthroline complexes with metal ions other than copper. Both chelating agents indicated in Table 2 block the 1,10-phenanthroline-cupric ion catalyzed aerobic oxidation of thiols in the same concentration range as they reverse the 1,10-phenanthroline inhibition of E. coli DNA polymerase I (10).

Similar data have been obtained with E. coli DNA dependent RNA polymerase and T-4 DNA polymerase and M. luteus DNA polymerase. In Tables 3 and 4, the data describing the reversal of the 1,10-phenanthroline inhibition of E. coli

TABLE 4

Reversal of 1,10-Phenanthroline Inhibition
of T-4 DNA Polymerase by Chelating Agents

OP[a]	NC[b]		Ter[c]	
(M)	(M)	%‡	(M)	%‡
0	10^{-5}	105	10^{-5}	124
0	10^{-4}	103	10^{-4}	124
0	10^{-3}	63	10^{-3}	107
$5X10^{-5}$	0	0	0	0
$5X10^{-5}$	10^{-5}	61	-	-
$5X10^{-5}$	10^{-4}	123	-	-
$1.5X10^{-4}$	0	0	0	0
$1.5X10^{-4}$	-	-	10^{-5}	2
$1.5X10^{-4}$	-	-	10^{-4}	88

See legend for Table 3.

RNA polymerase and T-4 DNA polymerase, respectively, by 2,2',2"-terpyridine and 2,9-dimethyl-1,10-phenanthroline are presented.

Discussion. These studies demonstrate that the 2:1 1,10-phenanthroline-cuprous ion complex which had been previously demonstrated to be an effective inhibitor of Pol I of E. coli is also a potent inhibitor of a variety of other polymerases. Since the total concentration of copper ion in all its forms does not exceed 1 μM, this coordination complex is a remarkably specific inhibitor. These observations clearly indicate that the reversible inhibition of these polymerases by 1,10-phenanthroline does not reflect the coordination of an active site zinc ion in the manner which has been demonstrated for horse

liver alcohol dehydrogenase by crystallographic and spectroscopic studies (11-14). At present no evidence is available which bears on the mechanistic function, if any, of the tightly bound zinc ions found in polymerases.

The reversible inhibition of an enzyme by 1,10-phenanthroline via its cuprous complex represents a novel mode of inhibition by this chelating agent. The 1,10-phenanthroline-cupric ion complex inhibits aldolase, among other enzymes, irreversibly by oxidizing essential sulfhydryl groups (15). In addition to being irreversible, this mode of inhibition is not dependent on the addition of exogenous thiol. The precise mechanism of the inhibition of these polymerases by the 1,10-phenanthroline-cuprous ion complex is as yet unknown. In view of the generality of the phenomenon, this question seems worthwhile to answer. If it is the result of the chance complementarity of the 2:1 1,10-phenanthroline-cuprous ion complex to the active site of these polymerases, it is somewhat surprising that the ortho methyl groups of 2,9-dimethyl-1,10-phenathroline generate sufficient steric hindrance to prevent the stable complex this ligand forms with cuprous ion from being an effective inhibitor.

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