DNA POLYMERASE OF PHAGE T4 ANTIMUTATOR MUTANT CB121

A defective zinc metalloenzyme?

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Received 24 May 1978

1. Introduction

Most, if not all DNA polymerases are zinc metalloenzymes [1–5]. The bivalent zinc ion is supposed to coordinate the 3′-OH group of the DNA primer and to promote its nucleophilic attack on the α-phosphorous of the deoxynucleoside triphosphate [5,6], a model based on experiments with *E. coli* DNA polymerase I [5]. The presence of zinc in a number of other DNA polymerases, including phage T4 DNA polymerase, is also mentioned in [5].

We have studied the inhibition and the inactivation of phage T4 DNA polymerase (the wild-type enzyme and two enzymes from temperature-sensitive mutants in the polymerase gene (gene 43)) by the zinc chelator 1,10-phenanthroline. The greater sensitivity of the antimutator mutant CB121 DNA polymerase compared to the wild-type and the mutator mutant L98 DNA polymerase is evidence for a disturbed interaction between the CB121 DNA polymerase and its metallic cofactor zinc. This is the first report of such a defect in a mutant DNA polymerase.

2. Materials and methods

All DNA polymerase preparations were from phage-infected *E. coli* B cells. Phage $T4^+$ from our laboratory and T4amN82 (gene 42) kindly supplied by Dr Goldfarb, Moscow, were used for the purification of wild-type DNA polymerase. The gene 43 ts-mutants L98 and CB121, classified as mutator and antimutator, respectively, in [7,8] were a generous gift from Dr Wood, Pasadena.

The T4 DNA polymerases were purified by the procedure in [9] via DEAE and phosphocellulose chromatography. Our preparations were free of *E. coli* DNA polymerase I as judged by their sensitivity to 1 mM *p*-hydroxymercuribenzoate (their inhibition exceeded 95% while *E. coli* DNA polymerase I (Boehringer, Mannheim, grade II) was unaffected).

DNA polymerase activity was assayed at 30°C according to [10]. The incubation mixture contained: 67 mM Tris–HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 6.7 μM EDTA, 10 mM 2-mercaptoethanol, 167 μg per ml BSA, 0.033 mM of each dATP, dCTP, dGTP and dTTP plus [^{3}H]dTTP and 0.2 mM denatured salmon-sperm DNA. In experiments with phenanthroline and zinc, these substances were included in the mix and all reactions were started by addition of the enzyme.

Substances: 1,10-phenanthroline – one lot (analytical grade) was from REANAL, Hungary, the other was synthesized and kindly given to us by Professor Madeja, Greifswald. $ZnSO_4 \cdot 7\ H_2O$ – one lot (analytical grade) was from VEB Laborchemie Apolda, the other ('specpure') from Johnson, Matthey and Co., London. Results with both lots of phenanthroline and $ZnSO_4$, respectively, were essentially identical.

3. Results and discussion

When first attempting to purify the T4 mutant CB121 DNA polymerase we were faced with very low enzyme activities in the cell extracts. We incorporated 0.1 mM $ZnSO_4$ in the medium of infected cells, following [11] where the activity of phage T7 RNA poly-

Table 1
Effect of 0.1 mM $ZnSO_4$ in the incubation medium of T4-infected cells on DNA polymerase activity in cell extracts

Exp. no.	Phage	Polymerase activity (units/ml)[a]	
		Incubation without zinc	Incubation with zinc
1	CB121	0.05	0.27
2	CB121	0.16	0.29
3	$T4^+$	10.4	5.2
4	$T4^+$	1.9	1.2
	CB121	0.9	2.0

[a] Definition of units is according to [9]

Equal volumes of *E. coli* B cultures grown in a defined medium based on phosphate buffer and casamino acids [10] were distributed into flasks with or without the appropriate amount of $ZnSO_4$ in aqueous solution and immediately infected with phage at 30°C. Conditions of infection and cell extraction were as in [9], the assay was as in section 2 except that 1 mM NaF [9] was contained in the assay mix. Parallel samples from one experiment were treated identically; differences between independent preparations are due to different dilution of extracts

merase (also a zinc metalloenzyme) could be enhanced by adding zinc during different purification steps. This led to an increase in the activity of CB121 DNA polymerase on the one hand, and to a reduction in the activity of the wild-type enzyme on the other (table 1).

The activation of CB121 DNA polymerase in the infected cell by zinc ions indicated that this mutant enzyme was defective in its capacity to bind zinc. We therefore tested the effect of the zinc chelator 1,10-phenanthroline on the purified DNA polymerases of $T4^+$, CB121 and L98, a mutant which displayed normal enzyme activity in extracts of infected cells. Figure 1 shows dose–effect curves with the inhibitor; clearly, CB121 DNA polymerase is the most sensitive of the three, while L98 DNA polymerase is slightly less affected than the wild-type enzyme. The inhibited reactions were linear with time and were not influenced by the addition of $MgCl_2$ equimolar to phenanthroline. At 42°C all three enzymes were about twice as sensitive to the chelator than at 30°C (data not shown).

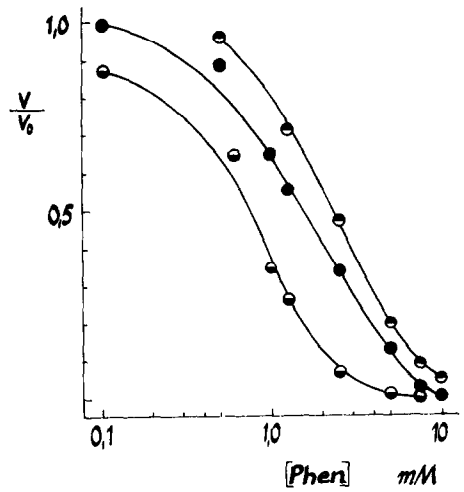

Fig.1. Inhibition of $T4^+$, L98 and CB121 DNA polymerase by 1,10-phenanthroline. $T4^+$ (●), L98 (⊖), CB121 (◐). v/v_0 = rate of the inhibited reaction divided by rate of the control.

The inhibition of a zinc metalloenzyme by a chelator must involve the formation of a ternary complex between the enzyme, the zinc ion and the chelator, which may either be stable or decompose rapidly into the metal-free apoenzym and the zinc–chelator compound. Which of these alternatives holds true in the case of T4 DNA polymerase could be decided by dialysis against 1,10-phenanthroline, a treatment which should remove zinc ions bound to the enzyme. Such experiments proved to be extremely difficult with T4 DNA polymerase, since the enzyme, while being remarkably insensitive to 1,10-phen-

Table 2
Dialysis of T4$^+$, L98 and CB121 DNA polymerase against 10 mM, 1,10-phenanthroline

polymerase of phage	% Residual activity after dialysis against		B/A X 100
	A buffer	B phenanthroline	
T4$^+$	59	56	95
L98	49	48	98
CB121	48	11	23

0.2 ml of each polymerase dilution containing 50 mM Tris–HCl (pH 8.0), 25% glycerol, 1 mg BSA/ml and 0.01 M 2-mercaptoethanol plus 0.2 mM denatured salmon-sperm DNA were dialyzed for 24 h at 4°C against dialysis buffer (50 mM Tris–HCl (pH 8.0), 25% glycerol, 1 μl 2-mercaptoethanol/ml) with or without 10 mM 1,10-phenanthroline. Dialyzed samples were diluted 1:22 into assay mix; assays were done in quintuplicates, so that standard deviations were kept below 10% in every case

anthroline under standard dialysis conditions compared with all other DNA polymerases investigated so far [4], is quite unstable towards dialysis per se. We therefore used very high phenanthroline concentrations – 10 mM instead of 1 mM used [4,5] and, this was decisive, denatured salmon-sperm DNA (0.2 mM) was added to the enzyme in the dialysis tubing. Under these specific conditions CB121 DNA polymerase was inactivated much more rapidly than the other two polymerases (table 2). If DNA was omitted, the CB121 DNA polymerase was so sensitive to dialysis in itself that the inactivation by phenanthroline could not be accurately estimated (data not shown).

The result of the dialysis experiment proves that CB121 DNA polymerase is indeed disturbed in its zinc binding capacity. However, all three T4 DNA polymerases are much more stable to 1,10-phenanthroline than, for instance, human lymphocyte DNA

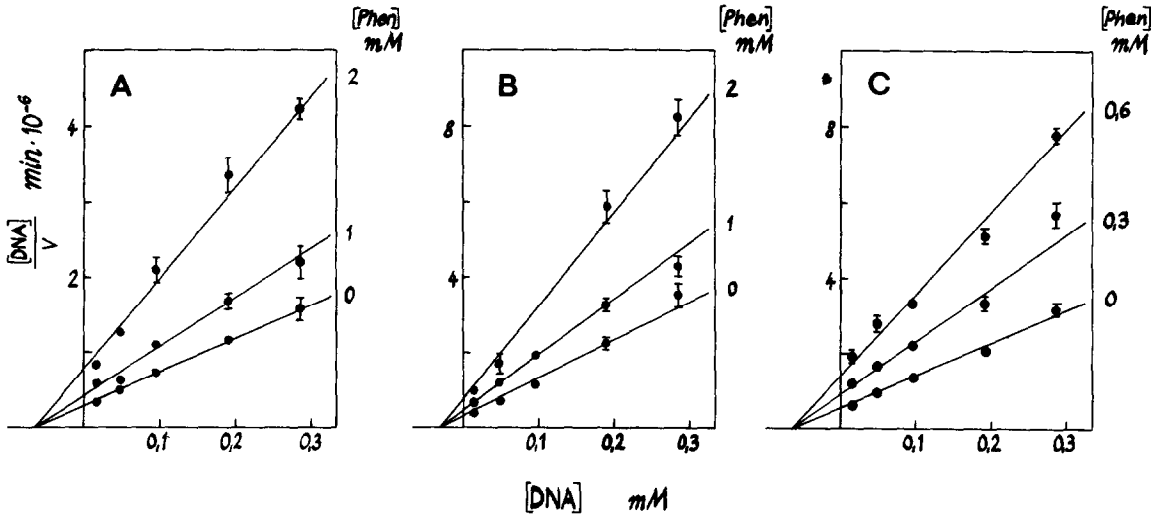

Fig.2. Dependence of inhibition by 1,10-phenanthroline on DNA concentration. The product of DNA concentration and reciprocal reaction rate (rate = pmol thymidine triphosphate incorporated/min) is plotted against DNA concentration (Hanes plot). 1,10-phenanthroline concentration (abbreviated Phen) is indicated in the figures. A, T4$^+$ DNA polymerase; B, L98 DNA polymerase; C, CB121 DNA polymerase.

polymerase or *E. coli* DNA polymerase I [4]. The slow inactivation during dialysis excluded loss of zinc as the mechanism of phenanthroline inhibition, i.e., the inhibited enzyme species is probably the ternary complex polymerase–zinc–phenanthroline.

Since the polymerase-bound zinc ion is supposed to coordinate the 3′-OH group of the DNA primer [5,6] it was of interest to see what effect varying the DNA concentration would exert on inhibition by phenanthroline. The results are shown in fig.2. The fact that the Hanes plots intersect on the abscissa indicates a noncompetitive mode of inhibition for all three enzymes. This corresponds to the mode of inhibition observed with AMV reverse transcriptase [4] and is opposed to the competitive inhibition observed with terminal deoxynucleotidyl transferase [12] and also indicated for *E. coli* DNA polymerase I and sea urchin DNA polymerase [1]. Since inhibition patterns of multisubstrate enzymes cannot be interpreted in a straightforward way [13] the noncompetitive inhibition of T4 DNA polymerase by phenanthroline does not contradict the model according to which both the chelator and DNA interact with the zinc ion. Besides that, the coordination sites occupied by the 3′-OH group of the primer and the two nitrogen atoms of phenanthroline might not be equivalent. This would help to explain why the sensitivities to the chelator and the Michaelis constants for DNA of the three DNA polymerases are not correlated (K_s = 0.064 mM for $T4^+$ and CB121 DNA polymerases; K_s = 0.029 mM for L98 DNA polymerases; data from two independent experiments).

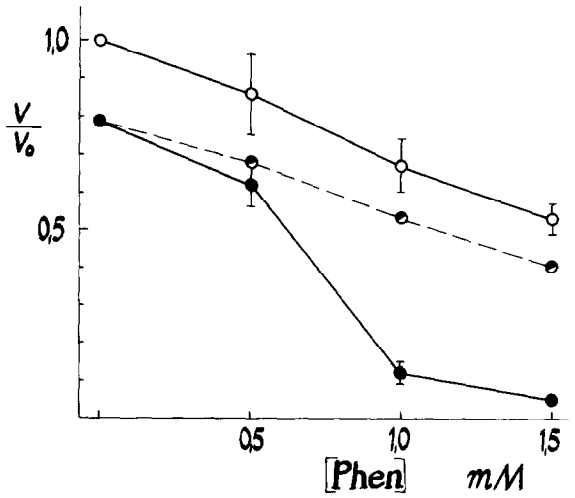

Fig.3. Inhibition of $T4^+$ DNA polymerase by 1,10-phenanthroline in the presence of zinc. Inhibition by phenanthroline alone (○); inhibition by phenanthroline in the presence of 0.5 mM $ZnSO_4$ (●); estimated values assuming that inhibition by phenanthroline + zinc were additive (⊖). v/v_0 = rate of the inhibited reaction divided by the rate of the control.

While the phenanthroline inhibition of terminal transferase is reversed by the addition of equimolar zinc [12] this was not the case with T4 DNA polymerase (fig.3). Zinc sulphate alone inhibits the enzyme, probably via template distortion [14], and the higher the phenanthroline-to-zinc ratio, the more synergistic the resulting inhibition. This synergism, perhaps reflecting several template inactivation by bulky phenanthroline molecules attached to DNA-bound zinc ions (a mixed RNA–Fe^{2+}–phenanthroline complex has been described [15]) makes it impossible to judge whether the chelator is released from the complex with the enzyme or not.

Our results indicate that the CB121 mutant DNA polymerase is defective in its binding to zinc. Since CB121 is an antimutator mutant [7] and the DNA polymerase purified from the allelic [8] mutant CB120 is more accurate than the wild-type enzyme [16], antimutagenicity and defective zinc binding might be related. Phage T4 DNA polymerase has been shown to require an activation step of unknown nature before gaining its catalytic power [17]. This step is temperature-sensitive in many ts-mutants of gene 43, including CB121. In view of the enhancement of CB121 polymerase activity achieved by adding zinc to the medium of infected cells, the conversion from apoenzyme to metalloenzyme might have something to do with the elusive activation step.

Acknowledgement

We thank W. Seipelt for reliable technical assistance.

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