ZINC IN DNA POLYMERASES

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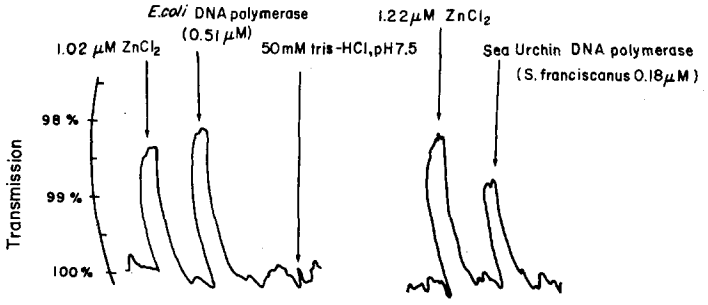
SUMMARY

Homogeneous DNA polymerases from E. coli and sea urchins contain zinc in a proportion of approximately 2 and 4 gm atoms Zn/mole enzyme respectively. Specific inhibition of the enzyme by ortho-phenanthroline and lack of inhibition by meta-phenanthroline suggest that the bound zinc plays a functional role in the interaction of DNA with the polymerases.

It has long been known that an added divalent cation, such as Mg^{++}, is required for DNA polymerase activity in vitro. Recently, Chang and Bollum have obtained evidence that the enzyme terminal deoxynucleotidyltransferase may, in addition to a cofactor requirement for Mg^{++}, utilize a tightly bound cation, probably zinc (1). The similarities in catalysis between the terminal transferase and DNA polymerase led us to analyze the latter for the presence of zinc. In vivo experiments (2) and nutritional studies (3-5) have also implied a role for zinc ions in DNA synthesis.

E. coli DNA polymerase was prepared by the method of Jovin et al. (6) and an additional phosphocellulose fractionation was performed on the final Fraction 7. This resulted in a protein preparation which was homogeneous, as judged by polyacrylamide gel electrophoresis. Sea urchin DNA polymerase was purified from nuclei of Strongylocentrotus franciscanus blastulae as previously described (7). Prior to analysis for zinc, all enzyme samples were dialyzed exhaustively, using a vacuum concentrating apparatus against zinc-free 50 mM tris HCl buffer, pH 7.5. The results of the analyses (Fig. 1) show approximately 2 gm atoms of zinc per mole of E. coli polymerase and 4 gm atoms of zinc per mole of sea urchin nuclear polymerase. E. coli



Protein and Source	Zinc Content gm atoms Zn/mole protein
E.coli DNA polymerase prep A_1	1.78
A_2	2.48
B	1.68
S. franciscanus Nuclear DNA polymerase prep A	4.36
B	4.01
E.coli Exonuclease III	0.11
Ribonuclease A	0.01
Bovine Serum Albumin	0.02

Fig. 1. Zinc content of homogeneous proteins measured by atomic adsorption spectroscopy. Above: output trances from atomic absorption spectrometer, showing zinc content of DNA polymerases and the noise levels encountered. The zinc analyses were performed using a Varian Techtron instrument coupled to an Aztec Log expander and an Esterline Angus recorder. All analyses were carried out with a noise level equivalent to 0.10 - 0.15 μM Zn^{++}.

Preparation A_1 of E. coli DNA polymerase (0.9 μM enzyme) was prepared as described in the text. Preparation A_2 (1.3 μM enzyme) was previously used in NMR studies (13) and has been repurified by vacuum dialysis (see below) and sucrose density gradient centrifugation (see Fig. 2). Preparation B (3.6 μM enzyme) was prepared by an earlier published method (8). The sea urchin preparation A (0.18 μM enzyme) and B (0.11 μM enzyme) were made at different times from nuclei of Strongylocentrotus franciscanus by the method of Loeb (7). E. coli exonuclease III (5.7 μM protein) was obtained during the preparation of DNA polymerase preparation A. The molecular weights were taken to be 109,000 for E. coli DNA polymerase (8); 150,000 for the sea urchin enzyme (7) and 30,000 for exonuclease III from its behavior on Sephadex G-100. Molarities were calculated from protein analyses. The specific activity of the E. coli DNA polymerase was 24,900 units/mg protein, and that of the sea urchin enzyme, 800-1,200 units/mg protein (1 unit is that activity which will incorporate 10 nmoles total deoxynucleotides into the primer in 30 min at 37° (8). "Activated" DNA (7) was the primer used.

Dialysis was carried out using a Schleicher & Schuell vacuum concentrating apparatus. The enzyme was put into the collodion bag and concentrated under vacuum against 50 mM tris HCl (Sigma Chemical Co. Trizma base), pH 7.5. When the final volume was approximately 0.1 ml the concentrated enzyme solution was then diluted with 5-10 ml of the same tris buffer and again subjected to vacuum dialysis. This was repeated 3 times. The buffer solution was contaminated with less than 0.1 μM Zn^{++}.

exonuclease III, which co-purified with DNA polymerase until the final step in purification (6) contained only contaminant amounts of zinc.

The chelating agent ortho-phenanthroline inhibits various DNA polymerases when the concentration of DNA is at the K_m concentration for each polymerase (Table I). However, no inhibition of the polymerases is observed with the meta- isomer of phenanthroline at concentrations as great as 1 mM. The aromaticity and the inhibitory properties of the two isomers of phenanthroline are similar with the exception that m-phenanthroline cannot chelate metals while the o-derivative is able to do so effectively (9,10). In these experiments Mg^{++} was present in at least 10-fold excess over the chelating agents. At saturating levels of DNA (5 to 20 times the respective K_m values) the polymerases were much less susceptible to inhibition by o-phenanthroline. In other experiments the effect of E. coli DNA polymerase on the proton relaxation rate of water shows no evidence of a paramagnetic contribution suggesting the absence of such paramagnetic ions as Cu^{2+}, Mn^{2+}, Fe^{3+}, Co^{2+} and Ni^{2+}. The most direct explanation of these results is that DNA and the chelating agent compete for the enzyme-bound zinc. D. Brutlag and

Table I. INHIBITION OF DNA POLYMERASES BY O-PHENANTHROLINE AT K_m LEVELS OF DNA.

Polymerase Source	DNA Concentration in μM phosphorus	Concentration of o-phenanthroline required for 50% inhibition (μM)
E. coli	34	40
Sea urchin	136	380
Human lymphocytes	136	1000

E. coli and sea urchin DNA polymerase activity was determined as described in Refs. 6 and 7, respectively, with "maximally activated" calf thymus DNA (7) as a primer. Human lymphocytes were stimulated with phytohemagglutinin and the crude homogenate was assayed for polymerase activity as previously described (11). The K_m concentration of DNA in these assays was half of that concentration required to give maximal incorporation under the conditions used.

A. Kornberg (private communication) have also observed inhibition of E. coli DNA polymerase after prolonged dialysis with o-phenanthroline.

Two types of experiments with E. coli polymerase indicate that the enzyme-bound zinc is exchangeable with zinc ions in the buffers used.

i) When E. coli B were grown in a medium containing the radioactive isotope ^{65}Zn and DNA polymerase subsequently purified (6), all of the fractions containing the enzyme also contained ^{65}Zn. However, at the ultimate (seventh) step when the enzyme was passed through Sephadex G-100 in a buffer containing 0.1 M potassium phosphate, 0.1 M ammonium sulfate, pH 7.0, all of the ^{65}Zn became separated from the DNA polymerase and appeared in the position of inorganic zinc. However, after dialysis the enzyme was found to still contain approximately 2 gm atoms of unlabeled zinc/mole enzyme. Atomic absorption spectroscopy revealed that the buffer used for Sephadex chromatography contained contaminating zinc ions in an approximately 10-fold excess over that present in the DNA polymerase itself.

ii) Addition of ^{65}Zn in tracer amounts to the highly purified E. coli DNA polymerase has shown that the radioactive isotope becomes associated with the enzyme and travels with the enzyme when subsequently separated from unbound zinc by centrifugation in sucrose density gradients (Fig. 2). Furthermore, the coincidence between ^{65}Zn and polymerase activity indicate that the zinc is bound to the polymerase itself and not sequestered on a possible contaminating protein.

Our results show that zinc is present in DNA polymerase purified from both bacteria and animal cells and suggest a functional role for the metal in the enzyme catalysis. The possibility that the metal is a tightly-bound, stoichiometric contaminant in both E. coli and sea urchin polymerases, though not excluded, is considered unlikely, in view of the diverse sources, isolation procedures and the inhibitor studies.

In the mechanism of action of DNA polymerase the role of the added Mg^{++} appears to be the facilitation of the binding of the deoxynucleoside

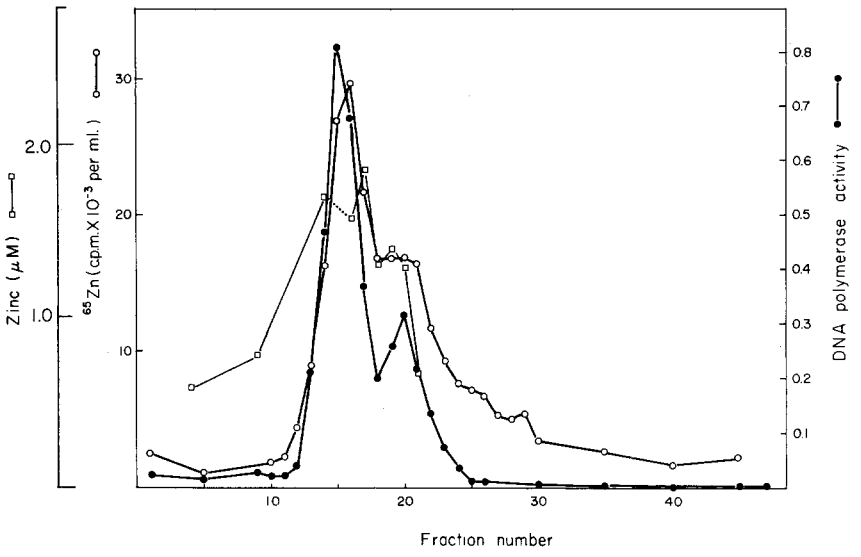


Fig. 2. Sucrose density gradient centrifugation of ^{65}Zn-labeled homogeneous E. coli DNA polymerase. Ten nmoles of enzyme were incubated at 0°C with 1 nmole of $^{65}ZnCl_2$ for 30 minutes. The enzyme was washed by vacuum dialysis and all further unbound $^{65}Zn^{++}$ removed by centrifuging through a 5-20 per cent (w/v) sucrose gradient containing 50 mM tris HCl, pH 7.5 (65 hr at 50,000 rpm, Spinco SW 40Ti rotor). Three nmoles of this enzyme preparation were then layered over 12.5 ml of a 5-20 per cent (w/v) linear sucrose gradient containing 50 mM tris HCl pH 7.5, and centrifuged for 40 hr at 40,000 rpm in the Spinco SW 40Ti rotor. The stock 20 per cent sucrose-tris HCl was passed several times through a column of Chelex 100 (neutral form) before use in these centrifugations, to reduce the contaminating zinc levels. ^{65}Zn concentration was determined on a gamma spectrometer and total zinc was determined by atomic absorption spectroscopy. DNA polymerase activity was determined on 3 μl aliquots of each fraction (7); incubation was for 5 min at 37° and activity is given in nmoles of dTM^{32}P incorporated into DNA. Since this enzyme preparation had been subjected to a number of manipulations it is likely that the shoulder on the peak corresponds to the active fragment produced by proteolytic cleavage (14,15).

triphosphate substrates to the polymerase, rather than binding of the DNA template (12). Analogous experiments with manganese using proton magnetic relaxation studies of water (13) support this view. Moreover, added zinc ions do not substitute for magnesium or manganese ions in the enzyme assay. It is therefore suggested that the tightly bound zinc ion is involved in the interaction of DNA with the enzyme. This view is supported by the insensitivity of various polymerases to ortho-phenanthroline inhibition at saturating levels of DNA. A relevant observation has been made with the

terminal deoxynucleotidyltransferase system (1), in which ortho-phenanthroline competitively inhibits the binding of oligodeoxynucleotide primers. The presence and exchangeability of zinc at the DNA binding site of DNA polymerase may provide a new probe for the investigation of DNA replication and may be of value in the design of chemotherapeutic agents for malignant disease. Thus a Zn chelating agent or a radioactive isotope of Zn could be brought to the site of DNA replication or repair and inhibit these processes.

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