THE ROLE OF Zn(II) IN TRANSCRIPTION BY T7 RNA POLYMERASE

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Summary: Homogeneous T7 RNA polymerase contains from 2-4 gm atoms of zinc per mole of M.W. 107,000. Inactivated molecules which can be separated from the active molecules by repeated chromatography contain less zinc, from 0.4 to 1 gm at per mole. Instability of the enzyme makes it difficult to relate maximal activity to a specific stoichiometry of Zn. The enzyme is inhibited by 1,10-phenanthroline, EDTA, CN^-, SH^-, N_3^- and by incubation with Chelex resin. Zinc is retained on gel filtration, but can be removed by dialysis for 96 hr against 5 mM 1,10-phenanthroline which totally inactivates the enzyme. Catalytic activity requires the presence of thiol reagents. Preparations with low activity can be activated by exogenous Zn ions.

A number of reports have implicated Zn(II) as a possible cofactor in a variety of nucleotidyl transferase enzymes including DNA polymerase I from E. coli (1), RNA polymerase from E. coli (2), and recently reverse transcriptase from avian myeloblastosis virus (3). We report here that the DNA dependent T7 RNA polymerase (product of gene-1 of phage T7), as isolated from phage-infected E. coli contains 2-4 gm atoms of Zn(II) per mole (M.W. 107,000). Under a variety of circumstances the enzyme can be activated specifically by Zn(II). In complex multicomponent systems like the polymerases using metal-nucleoside triphosphates as substrates, the precise role of a metal ion is difficult to prove. Initial experiments to delineate the role of Zn(II) in the T7 polymerase are presented here.

Materials and Methods

Enzyme.--T7 RNA polymerase was prepared from E. coli B (strain SY 106) (4) infected with a deletion mutant of T7(T7d14) (5). E. coli and phage were kindly supplied by Dr. William Summers. The enzyme was prepared by methods to be described elsewhere (6,7) and the procedure is outlined briefly below. The cells were broken with glass beads in a Waring blender, the extract was precipitated with polyethyleneimine (Pfaltz and Bauer, Inc., Flushing, New York), and the T7 polymerase extracted with dilute KCl, followed by chromatography on phosphocellulose. The enzyme was further purified by successive chromatography on hydroxyapatite and DEAE Sephadex. The final enzyme gave a single band on SDS gel electrophoresis migrating at a position corresponding to a molecular weight of ∿110,000 (8).

Assay.--The assay mixture contained 40 mM Tris HCl, 30 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.4 mM of each of the nucleoside triphosphates, [^{3}H]-ATP (6.25 μc/μmole, final specific activity), 12.5 μg/ml rifampicin and 50 μg/ml T7 DNA. Enzyme was added as a 5 μl aliquot from the desired stock. Enzyme concentrations will refer to the stock solution. Incorporation of [^{3}H]-ATP into RNA was measured by placing a 50 μl aliquot of the reaction mixture on a Whatman 3MM filter paper disc followed by precipitation with trichloroacetic acid. Discs were washed 4x for 10 minutes in 5% trichloroacetic acid, 1x with 50% ethanol, and 1x with diethyl ether. Discs were counted as previously described (9). T7 DNA was prepared by phenol extraction of phage purified by banding in a CsCl equilibrium density gradient.

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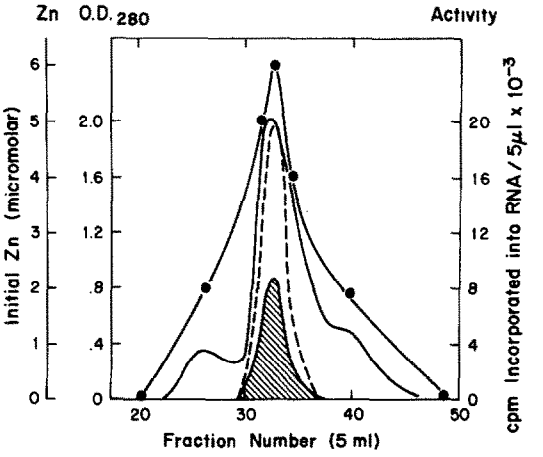

Figure 1. Hydroxyapatite chromatography of T7 RNA Polymerase. Zinc content (●); OD_{280} (—); activity before addition of Zn (▨); activity after addition of 0.1 mM Zn (----). Conditions: 0.1 M KCl, 5% glycerol, 0.01 to 0.35 M potassium phosphate gradient, pH 7.9.

Specific Activity is defined as nanomoles of ATP incorporated into RNA per hour per mg of enzyme at 37°.
Protein concentration was determined by absorbance at 280 nm using $E_{280}^{0.1\%} = 0.74$ (6).
Metal Analyses were performed by atomic absorption spectroscopy (10).

Results

Zinc Content of Purified T7 RNA Polymerase

Zinc is associated with the fractions containing rifampicin insensitive RNA polymerase activity throughout the purification procedure as illustrated here by the elution profile of a hydroxyapatite column used in the purification procedure (Figure 1).

In general the zinc concentration rises as the specific activity increases (Table I) Zinc content per mole tends to rise in the highly active preparations purified from inactive molecules by repeated chromatography (Table I). However, the enzyme is rather unstable and there is considerable variation in specific activity between different preparations. The zinc content of six different preparations of the polymerase after the final DEAE Sephadex chromatography are tabulated in Table II.

These RNA polymerase preparations are extremely active, on the order of 4×10^5 dAT units per mg of enzyme, compared to approximately 2×10^4 reported for the E. coli RNA polymerase (8). While the most active preparations contain 2 or more zinc ions, the enzyme is too unstable to draw conclusions as yet about the relationship between the number of zinc atoms found and activity. Zinc is only

Table I

A. Specific Activity and Zinc Content of T7 RNA Polymerase

Column	Total Activity cpm	% Recovery at each step	Specific Activity dAT units/mg	Zn gat mole
Phosphocellulose I	766,000		a	a
Phosphocellulose II	423,000		a	a
Hydroxyapatite I	250,000	33	56,000	0.19
Hydroxyapatite II	220,000	52	115,000	1.1
DEAE Sephadex II	146,000	66	400,000	2.0

a. Nonspecific optical density of effluent after polyethyleneimine treatment prevents meaningful calculation.

Table II

Zinc Content of Homogeneous DNA Dependent RNA Polymerase of Bacterophage T7

Enzyme Preparation	Activity dAT units/mg	Zinc Content gm at/mole*
I	56,000	2.3
II	28,000	1.1
III	40,000	3.7
IV	400,000	4.0
V	175,000	1.7
VI	400,000	2.0

*Based on a molecular weight of 107,000 and an $E_{280}^{0.1\%}$ of 0.74 calculated from the aromatic amino acid composition (6). Mn and Cu were below detection limits < 0.1 gm at/mole; Co = 0.3 gm at/mole in some preparations; Fe = 0.5 to 1.5 gm at/mole.

one of several factors which stabilize or enhance the activity of the enzyme which include glycerol and thiol reagents.

Activation of T7 RNA Polymerase by Zn(II) and Sulfhydryl Reagents, Inhibition by First Transition Metal Ions.--The zinc of the homogeneous T7 RNA polymerase is firmly attached to the enzyme and elutes with the enzyme on gel filtration over G-25 Sephadex (Figure 2A). The preparation shown retained a zinc content of between 3 and 4 gm atoms per mole. If gel filtration is performed on a column not equilibrated with sulfhydryl reagents, the zinc is retained, but the polymerase activity is lost (Figure 2B). Equilibration of the column with dithiothreitol preserves a large proportion of the activity, but even more is preserved if the column is equilibrated with both dithiothreitol and zinc (Figure 2B). Nevertheless the enzyme loses significant activity on gel filtration and dilution.

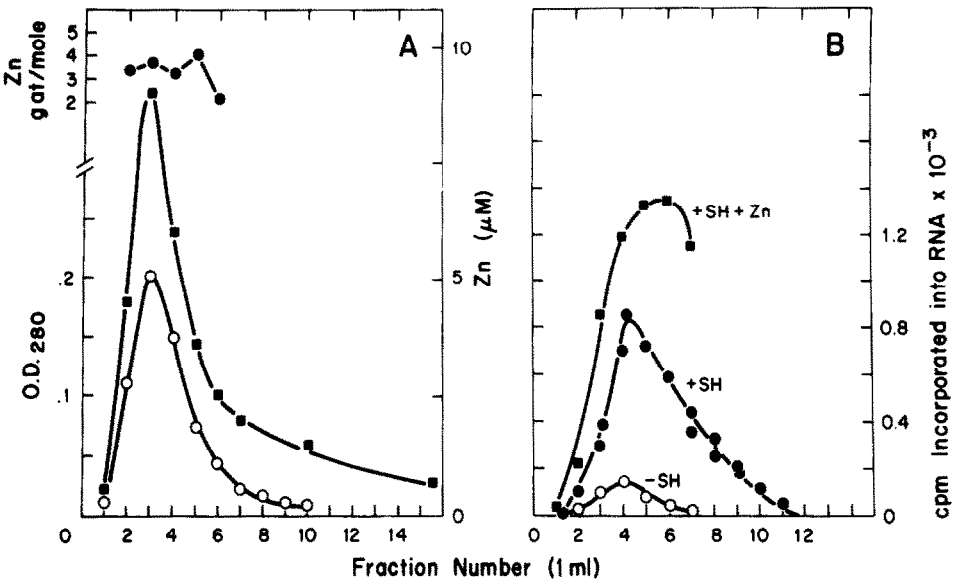

Figure 2. A. Gel filtration of T7 RNA polymerase. Conditions: G-25 Sephadex, 1 x 25 cm column, 1 ml of 3.5 x 10^{-6} M polymerase applied to the column. Conditions: 0.01 M Tris, 0.1 M KCl, 0.1 mM dithiothreitol, pH 7.9. Zinc (■), OD_{280} (○). B. Activity of T7 RNA polymerase after gel filtration under conditions of part A. (○) minus dithiothreitol; (●) column equilbrated with 0.1 mM dithiothreitol; (■) column equilbrated with 0.1 mM dithiothreitol, 10^{-5} M Zn(II).

During the preparation of the polymerase it was observed that a number of the preparations could be significantly activated by the addition of zinc (e.g. see Figure 1). A detailed analysis of this activation is given in Figure 3. Some preparations show increasing activation up to 10^{-3} M Zn(II) under normal assay conditions (Figure 3A). A few of the very active preparations were not activated by addition of exogeneous Zn(II) (Figure 3A). Whether this variation in activation by Zn(II) reflects variable dissociation of zinc from the enzyme is presently under investigation. The best preparations continue a high rate of nucleotide incorporation for 2 hours and longer (Figure 3A). Bovine serum albumin was omitted from all assays in order to simplify the system as much as possible. For those preparations activated by Zn(II), the activation is specific for Zn(II) among the first transition and IIB metal ions (Figure 3B). The inhibition by Mn(II) and Co(II), may relate to the inability of the enzyme to use the metal complexes of the nucleoside triphosphates with these metals ions. Like other nucleotidyl transferases the T7 RNA polymerase is a Mg^{++} activated enzyme (8) and the Zn^{++} effects are in addition to those of the activating Mg^{++} ion. In contrast to some RNA polymerases the T7 enzyme shows no activity with Mn^{++} as the activating metal ion (8).

Inhibition by Metal Complexing Agents.--The effect on activity of incubation of

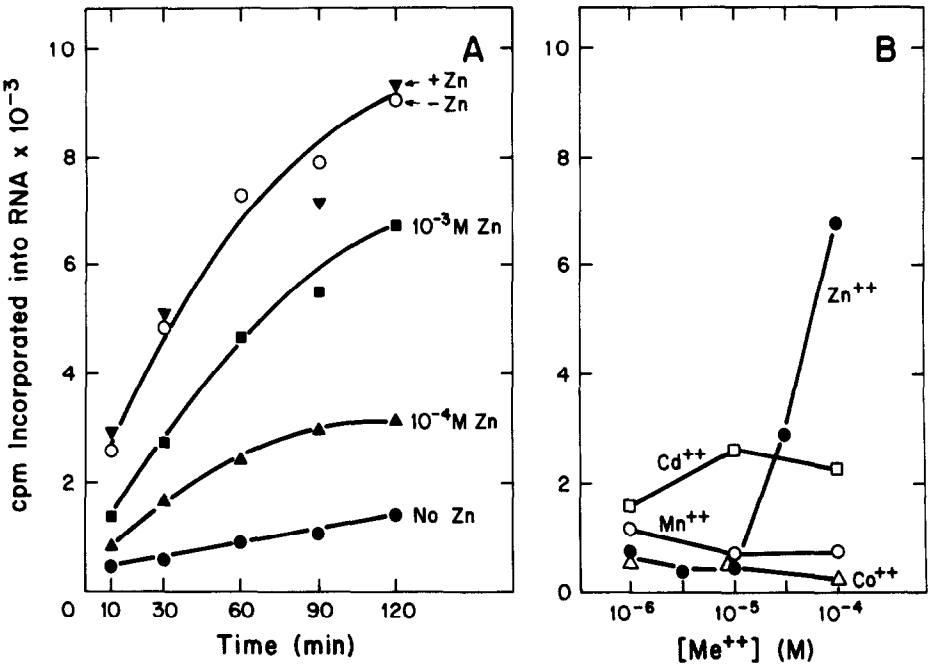

Figure 3. Activation of T7 RNA polymerase by exogenous zinc. Assays were as described in methods. Enzyme was incubated prior to assay with the metals and concentrations indicated on the figure. With the exception of the top curve in part A all the enzyme was from the same preparation. (▼, ○) in part A are assays of preparation VI (Table II) plus or minus exogenous Zn(II).

the enzyme with a variety of metal complexing anions and chelating agents is shown in Figure 4A. There is relatively little instantaneous inhibition of the enzyme by the metal complexing agents, but activity falls rapidly compared to the control incubated under the same conditions. Chelex resin, cyanide, and 1,10-phenanthroline are the most effective agents, reducing activity to 25% of that of the original enzyme in 4 hr, while the control has lost less than 10% of its activity. Equilibrium dialysis experiments are difficult to do with this enzyme, since it tends to lose activity on prolonged dialysis. Dialyses of two samples of enzyme (3×10^{-6} M), one against metal-free buffer and one against 5×10^{-3} M 1,10-phenanthroline are shown in Figure 4B. Zinc content and activity were determined at intervals during the 144 hr dialysis against a 500-fold volume excess. The sample dialyzed against 1,10-phenanthroline rapidly loses both Zn(II) and activity and all Zn(II) has been removed by 96 hr and the enzyme is completely inactive. Some of the zinc is lost from the enzyme dialyzed against metal-free buffer and it loses about 30% of its activity by 96 hr (Figure 4B).

Discussion

Highly purified DNA-dependent RNA polymerase of T7 clearly contains a

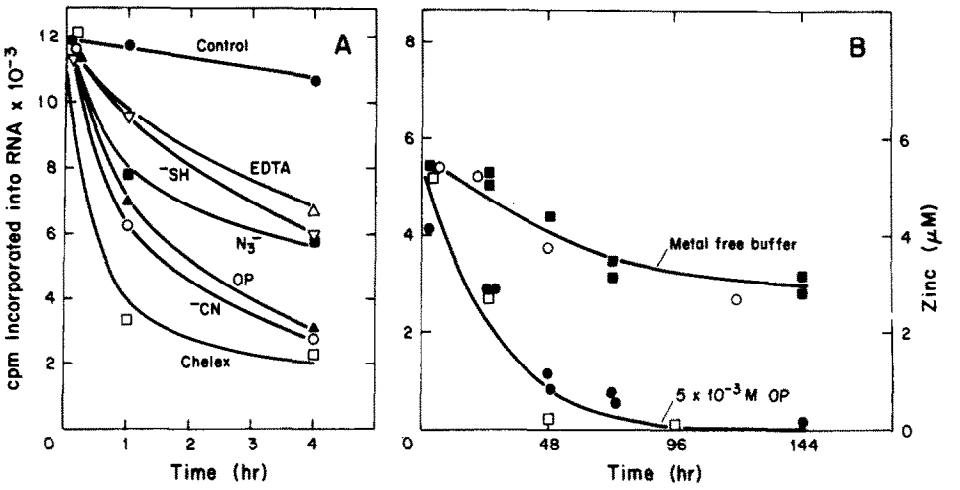

Figure 4. A. Inhibition of T7 RNA polymerase by EDTA (Δ), SH^- (▽), N_3^- (■), 1,10-phenanthroline (▲), CN^- (O), and Chelex (□). Enzyme, 3 x 10^6M, was incubated with mM inhibitor or layered over Chelex resin for the indicated time and assayed in the usual manner at 37°. Incubation conditions: 0.01 M TrisHCl, 0.1 M KCl, 0.1 mM dithiothreitol, pH 7.9, 4°. B. Equilibrium dialysis of T7 polymerase, 3 x 10^{-6} M, against metal-free buffer (■, activity, middle ordinate; O, zinc) and 5 mM 1,10-phenanthroline (● , activity; □ , zinc). Conditions; 0.01 M Tris, 0.1 M KCl, 0.1 mM dithiothreitol, pH 7.9, 4°. Inhibition by 1,10-phenanthroline is more rapid at 37°.

significant quantity of zinc which is not readily removed from the enzyme (Table II, Figures 2 and 4). Studies of the functional significance of the Zn(II) are hampered by the instability of the enzyme, since dilution and dialysis in the absence of glycerol or thiol reagents tend to inactivate the enzyme. Incubation of the enzyme with chelating agents inactivates the enzyme more rapidly than the control and loss of activity appears to coincide with the loss of zinc (Figures 3 and 4). The enzyme contains significant, but variable amounts of iron (Table II) which probably represents non-specific contamination, similar to that found in a number of other zinc metalloenzymes. The presence of small but significant amounts of cobalt is somewhat unusual and represents concentration of the ion because of the extremely low levels of cobalt in most biological systems. There is considerable variation in the amount of contaminating protein carried through the phosphocellulose and hydroxyapatite chromatography which accounts for considerable variation in Zn/protein ratio at the hydroxyapatite step (Figure 1, Table I).

Monitoring of the various column purification steps by gel electrophoresis of the fractions suggests that there is a good deal of inactive T7 polymerase associated with the active enzyme from the phosphocellulose column.

Chamberlin estimated that his preparations of the T7 polymerase may have contained only 20% active molecules (8). By repeated chromatography on hydroxyapatite and Sephadex we have succeeded in removing a large proportion of the inactive 107,000 molecular weight units, since they elute slightly behind the most active polymerase. For example, the peak in the hydroxyapatite column (Figure 1) contains many inactive 107,000 units as shown by repeated chromatography. The most active preparations obtained in this manner have a specific activity of 400,000 units per mg compared to the figure of 900,000 units per mg estimated by Chamberlin for the pure enzyme of 107,000 molecular weight continuously synthesizing RNA at a rate of 100 nucleotides per second. These most active preparations contain the most Zn(II) (Table I). Fractions containing large proportions of inactive 107,000 molecules were found to contain variable amounts of Zn(II) (0.4 to 1.5 gm at/mole) most frequently significantly below 1 gm at/mole. It is possible that removal of zinc is one of the factors leading to inactivation of the enzyme. The addition of relatively high concentrations of extra Zn(II) may successfully reactivate some of these molecules (Figure 3). The precise function of the Zn(II) must be approached with caution, since Zn(II) is known to have marked effects on the DNA template. Zn(II) has been shown to assist the reversible unwinding and rewinding of the DNA double helix of native DNA's induced by heating and cooling (11). Exogenous zinc can interact readily with sulfhydryl groups and there appear to be sulfhydryl groups on the enzyme required to be in the reduced state for function (Figure 2B).

E. coli grows normally in "zinc-free" media prepared by the usual means of extraction with dithizone in CCl_4 and Chelex resin (12). Under "zinc-free" conditions the zinc enzyme alkaline phosphatase shows only 5% of its normal activity, since most of the protein is produced as a zinc-free apoenzyme (12). This does not interfere with metabolism unless E. coli is forced to grow on a phosphate ester as the sole carbon source (12). Both DNA polymerase I and E. coli RNA polymerase are reported to contain zinc, yet normally achievable "zinc-free" conditions do not appear to interrupt their function. Likewise we have not been able to interfere with T7 infection, maturation, and lysis by extraction of zinc from the growth medium. These failures to perturb function may, however, relate to the low level of zinc required for these enzymes compared to alkaline phosphatase. While alkaline phosphatase is reduced to 5 to 10% of its normal activity by "zinc-free" media the protein is present in the cell in many more copies (up to 6% of the total protein) than the DNA and RNA polymerases. By normal extraction procedures zinc can be reduced to a concentration of 10^{-8} to 10^{-7}M (12). This may still be sufficient to supply zinc for a number of essential E. coli enzymes if they are present in a limited number of copies per cell.

A number of functions for Zn(II) in transcription could be visualized including a structural role in the polymerase, alteration of the conformation of the DNA template, stabilization of the enzyme-template complex, or a catalytic function involving charge neutralization or electron withdrawal in a nucleophilic attack of the 3'-OH of the growing chain on the phosphorus of the incoming nucleotide. Proof of any of these mechanisms will require extensive work relating the zinc content of the T7 RNA polymerase to specific steps in the transcription process. We are currently investigating whether zinc has any effect on the nature or number of the in vitro transcripts. We have observed that Zn(II) can increase the efficiency of binding of T7 RNA polymerase to [^{3}H]-T7 DNA as assayed by the nitrocellulose filter assay.

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