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# Zinc Is Associated with the $\beta$ Subunit of DNA-Dependent RNA Polymerase of *Bacillus subtilis*†

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ABSTRACT: The *Bacillus subtilis* DNA-dependent RNA polymerase holoenzyme and core enzyme each contain approximately two atoms of zinc per molecule. When the dissociated subunits of the enzyme are passed through a blue dextran–Sepharose affinity column, only the $\beta$ subunit binds to the column. The total zinc content of the enzyme is tightly bound to the $\beta$ subunit. Dialysis studies suggest that the two zinc ions differ in the strength of their association with the $\beta$ subunit. The presence of zinc in $\beta$ is consistent with several other lines of evidence which indicate that this subunit is directly involved in phosphodiester bond formation. The blue dextran–Sepharose column procedure should be useful in future studies of the dissociation and reassociation of the enzyme since the method is rapid and provides excellent recovery of the $\beta$ subunit as well as the $\alpha$ and $\beta'$ subunits of the RNA polymerase.

Several nucleotidyl transferases have been reported to contain tightly bound zinc which is essential for their activity. These include DNA-dependent RNA polymerase from *E. coli* (Scrutton et al., 1971), phage T7 RNA polymerase (Coleman, 1974), yeast RNA polymerase I (Auld et al., 1976) and B (Lattke and Weser, 1976), DNA polymerase from *E. coli* (Slater et al., 1971; Springgate et al., 1973) and from sea urchin (Slater et al., 1971), and RNA-dependent DNA polymerase from avian myeloblastosis virus (Poesz et al., 1974; Auld et al., 1974). Several studies on other nucleotidyl transferases have suggested that they may be metalloenzymes since they are inhibited by chelating agents such as 1,10-phenanthroline (Williams and Schofield, 1975; Chang and Bollum, 1970; Valenzuela et al., 1973).

Our studies were undertaken to determine whether the RNA polymerase of *Bacillus subtilis* is a zinc metalloenzyme and, if so, where the metal is located with respect to the subunits of this oligomeric protein. It appeared that the latter question might be investigated by use of a blue dextran–Sepharose affinity column. As reported by Thompson et al. (1975), the dye blue dextran forms complexes with a large number of nucleotide-binding enzymes, which possess the structural feature

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termed the "dinucleotide fold." The enzymes which bind to blue dextran-Sepharose columns include DNA polymerase from *E. coli;* therefore, it seemed possible that the RNA polymerase from *B. subtilis* would bind to the column. This turned out to be the case; furthermore, methods were developed whereby the dissociated subunits of RNA polymerase could be separated on a blue dextran column and subsequently examined for their zinc content.

## Experimental Procedure

### *Materials*

All reagents were purest available commercial products, used without further purification except where noted. Tritiated UTP was obtained from Schwarz/Mann, unlabeled nucleoside triphosphates from Boehringer Mannheim, poly[d(AT)] from P-L Biochemicals, 1,10-phenanthroline from Aldrich, 1,7-phenanthroline from K & K (recrystallized from ethanol), super pure Tris[1] from Schwarz/Mann, and spectrograde glycerol from Eastman. Labware was acid washed and water was double distilled.

### *Methods*

*Growth Conditions and Media.* A clone of *Bacillus subtilis* 168 from an 18-h culture grown on a tryptose blood agar plate (Difco) was inoculated into 2 × SG medium (Leighton and Doi, 1971) and grown overnight at 37 °C with vigorous shaking. The cells from the overnight culture were serially transferred (5% inoculum) three times in very rich medium containing 2% yeast extract (Difco), 2.5% tryptose (Difco), 3% $K_2HPO_4$, and 3% glucose. The cells were grown at 37 °C with vigorous aeration and harvested during exponential growth at Klett readings of 600–750. The cells were resuspended and washed twice with buffer A and then stored at −70 °C until needed.

*Buffers.* Buffer A: 0.1 M Tris, 10 mM $MgCl_2$, 1 mM EDTA, 0.3 mM dithiothreitol, 1 M KCl, 2 mM phenylmethanesulfonyl fluoride, 10% glycerol, pH 7.9 at 4 °C. Buffer C: 20 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA, 0.2 mM dithiothreitol, 2 mM phenylmethanesulfonyl fluoride, 20% glycerol, pH 7.9 at 4 °C. Buffer E: 50 mM Tris, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1 mM phenylmethanesulfonyl fluoride, 10% glycerol, pH 7.9 at 4 °C. Buffer F: 20 mM Tris, 10 mM $MgCl_2$, 0.2 mM dithiothreitol, 6.5 M urea, 10% glycerol, pH 7.9 at 4 °C. Buffer G: 20 mM Tris, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 1 M $NH_4Cl$, 20% glycerol, pH 7.9 at 4 °C.

*Purification of RNA Polymerase.* The method of Fukuda and Doi (1977) was used for the isolation of the holoenzyme and core enzyme from 50 g of cells. For larger preparations, the method was modified; after the dextran–poly(ethylene glycol) step, the enzyme preparation was applied to a 5 × 6.5 cm column of heparin–Sepharose 6B (Sternbach et al., 1975) equilibrated with buffer C + 0.1 M KCl. The column was washed with the same buffer before the enzyme was eluted with buffer C + 0.75 M KCl. This step removed much of the extraneous protein present in large preparations. Subsequently, the enzyme was chromatographed on DEAE-cellulose and DNA-cellulose as in the method of Fukuda and Doi (1977), except that the buffers contained 20% glycerol and the column equilibration buffers contained 0.1 M KCl. These modifications led to a much higher yield of holoenzyme relative to core enzyme. As determined by $NaDodSO_4$ gel electrophoresis, the enzyme was greater than 98% pure after chromatography on DNA-cellulose.

To prepare core enzyme, the holoenzyme was dialyzed against buffer E + 20 mM KCl for 24 h and then applied to a 1.6 × 22 cm phosphocellulose column equilibrated with the same buffer. The σ factor was eluted in two column volumes of this buffer, and then the core enzyme was eluted with buffer E + 0.6 M KCl. The core enzyme was then dialyzed against buffer C + 0.6 M KCl.

*Separation of the Subunits of the Core Enzyme.* A solution of 20 mg of core enzyme in 7.5 mL of buffer C + 0.6 M KCl was mixed with an equal volume of deionized 9 M urea and sufficient dithiothreitol for a final concentration of 10 mM. After 10 min at 0 °C, the mixture was dialyzed against buffer F for 2 h and then applied to a 1.6 × 15 cm blue dextran–Sepharose 4B column (Thompson et al., 1975) equilibrated with buffer F. The column was washed with this buffer until the effluent absorbance at 280 nm fell to zero. The initial effluent contained the α and β′ subunits. The β subunit was eluted with buffer F + 0.6 M KCl. The subunits were then dialyzed against buffer G.

*Polyacrylamide Gel Electrophoresis.* The urea–$NaDodSO_4$ system of Wu and Bruening (1971) was employed.

*Protein Determination.* Protein concentrations were determined by the method of Lowry et al. (1951) or by measuring absorbance at 280 nm; a solution of 1.6 mg/mL of RNA polymerase in a 1-cm cell had an absorbance of 1.0.

*Zinc Analysis.* Atomic absorption measurements were made with a Perkin-Elmer Model 303 instrument, using the zinc absorption line at 214 nm. Before use, all labware was soaked in 1:1 $HNO_3$–$H_2SO_4$ to remove metal ions (Thiers, 1957). Buffers were made with the purest available reagents and were found to contain very little zinc (0–40 ppb). Measurements were standardized by adding known amounts of zinc to the experimental buffer. Each data point consists of the average of three 2-s integral measurements on a given sample; the numbers in Table I are averages of several such data points. Before analysis each sample was dialyzed for 2 days at 4 °C against a buffer containing 1 mM EDTA. To avoid contaminating the sample, analysis was usually performed by severing the dialysis bag with a clean scalpel blade and inserting the aspirator tube directly into the bag. Typical solutions of the holoenzyme or of the β subunit contained 100–250 ppb zinc (0.8–3.8 μM protein); all other solutions contained 0–40 ppb zinc.

## Results

The elution of RNA polymerase from a DNA-cellulose column is shown in Figure 1; two peaks of activity are evident. The first peak which has the lower specific activity contains the core enzyme; the second peak contains the holoenzyme. The $NaDodSO_4$–polyacrylamide gel patterns of the peak fractions are shown in Figure 2, indicating that the preparations were more than 98% pure. Analogous to the RNA polymerase of *E. coli,* the largest polypeptide chain is designated as β′; the second largest is designated β. As shown in Table I, atomic absorption analysis revealed that the holoenzyme contains two atoms of zinc per molecule and that the core enzyme likewise contains two atoms of zinc per molecule; thus it is highly unlikely that the σ factor contains zinc. It should be emphasized that the zinc content which we discuss here is quite firmly associated with the enzyme. Besides remaining attached during purification, the zinc remains bound during

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

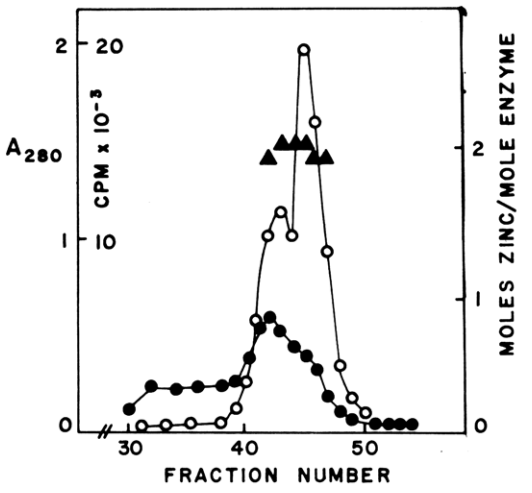


FIGURE 1: The elution pattern of *B. subtilis* RNA polymerase holoenzyme and core from a DNA-cellulose column. The RNA polymerase was eluted by a KCl gradient from 0.3 to 1.0 M. Each fraction (3.6 mL) was analyzed for $A_{280}$ (●) and assayed for RNA polymerase activity (○). The zinc content of the fractions was determined by atomic absorption and is plotted as the atoms of zinc per molecule of RNA polymerase (▲). Fractions 44 and 46 represent the peak activities of core and holoenzyme, respectively.

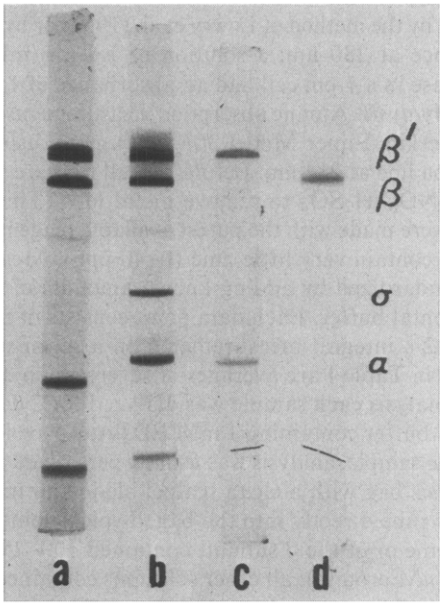


FIGURE 2: Typical NaDodSO$_4$–polyacrylamide gel electrophoresis patterns of RNA polymerase holoenzyme, core, and subunits. (a) Core enzyme, fraction 43, from Figure 1. (b) Holoenzyme, fraction 46, from Figure 1. (c) $\alpha$ and $\beta'$ subunits, fraction 11, from Figure 3. (d) $\beta$ subunit, fraction 41, from Figure 3.

TABLE I: The Zinc Content of Holoenzyme, Core Enzyme, and Subunits of RNA Polymerase from *Bacillus subtilis*.

| Preparation | Molar ratio of zinc to protein | Chromatography used in isolation or conditions used in dialysis |
|---|---|---|
| Core | 1.97 ± 0.06 (3)[a] | DNA-cellulose[b] |
| Holoenzyme | 2.02 ± 0.14 (3) | DNA-cellulose[b] |
| $\beta$ | 1.98 ± 0.14 (5) | Blue dextran[c] |
| $\alpha,\beta'$ | 0.01 ± 0.01 (5) | Blue dextran; 1 mM EDTA for 2 days[c] |
| $\beta$ | 1.07 | Blue dextran; 1 mM EDTA for 9 days[d] |
| $\alpha$ | 0.02 | Phosphocellulose in the presence of urea[e] |
| $\beta'$ | 0.03 | Phosphocellulose in the presence of urea[f] |
| Core + holoenzyme | 1.95 ± 0.05 (4) | DNA-cellulose; 10 or 20% glycerol and 0.1 or 1.0 mM EDTA for 7 days |
| Core + holoenzyme | 2.01 | DNA-cellulose; 10 mM EDTA for 1 day |
| Core + holoenzyme | 0.98 | DNA-cellulose; 10 mM EDTA for 7 days[g] |
| Holoenzyme | 1.98 | DNA-cellulose; 1 mM 1,10-phenanthroline for 1 day |

[a] The numbers in the parentheses indicate the number of samples analyzed by atomic absorption; three 2-s integral readings were taken on each sample. [b] See Figure 1. [c] See Figure 3. [d] Fraction 41, Figure 3. [e] The concentration of $\alpha$ was 13.3 $\mu$M. [f] The concentration of $\beta'$ was 1.2 $\mu$M. [g] Specific activity after dialysis was approximately 20% of the control value (dialysis vs. 1 mM EDTA).

prolonged (7 days) dialysis against 1 mM EDTA. The possibility remains that the enzyme is more weakly associated with other metal ions which are removed during purification; however, the enzyme activity is not altered by dialysis against 1 mM EDTA. When the enzyme is dialyzed against 10 mM EDTA for periods longer than 24 h, a significant amount of zinc is removed. The dialysis of the enzyme for 7 days against 10 mM EDTA led to removal of 50% of the zinc and loss of approximately 80% of the specific activity of the enzyme (Table I).

*Chromatography of RNA Polymerase on Blue Dextran–Sepharose.* It was found that RNA polymerase could be separated from a partially purified mixture by binding it to blue dextran–Sepharose (Halling and Doi, unpublished). The enzyme could be eluted from the column with a buffer containing either 0.12 M KCl or 10–15 mM UTP (or ATP). The concentration of nucleoside triphosphate required for elution of the enzyme is an order of magnitude lower than the minimum concentration of KCl required for elution; this marginally satisfies the criteria of Thompson et al. (1975) for specific binding to blue dextran–Sepharose.

When RNA polymerase core enzyme was dissociated into subunits in 6.5 M urea and was chromatographed on blue dextran–Sepharose, the $\beta$ subunit bound to the column while the $\alpha$ and $\beta'$ subunits did not. The $\beta$ subunit was eluted by the addition of 0.6 M KCl. A typical elution pattern is shown in Figure 3; NaDodSO$_4$–polyacrylamide gels of the two protein peaks are shown in Figure 2. This chromatographic procedure is relatively rapid and provides the subunits in good yield.

*Zinc Content of the RNA Polymerase Subunits.* Results of atomic absorption analysis of the separated subunits are presented in Table I and in Figure 3. After dialysis against 1 mM EDTA for 2 days to remove extraneous metal ions, the solutions containing the $\beta$ subunit showed the presence of approximately two atoms of zinc per subunit; solutions containing the $\alpha$ and $\beta'$ subunits contained practically no zinc. Isolation of the $\alpha$ and $\beta'$ subunits by chromatography on phosphocellulose (Lill et al., 1975), followed by zinc analysis, led to the same conclusion. In contrast to the *E. coli* enzyme, the $\beta'$ subunit of *B. subtilis* eluted from the phosphocellulose column at a lower ionic strength (0.16 M KCl) than $\beta$ (0.20 M KCl). One sample containing the $\beta$ subunit was dialyzed against 1 mM EDTA for a total of 9 days, after which it was found that the sample contained one atom of zinc per subunit. Taken to-

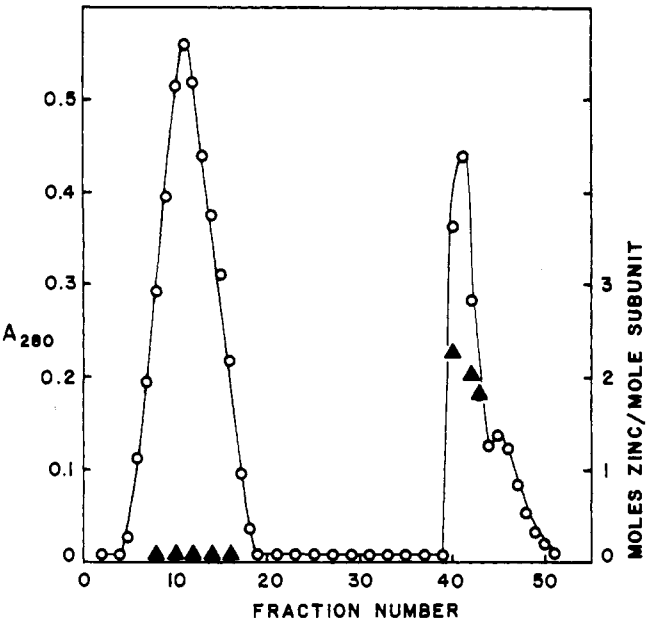


FIGURE 3: Typical elution pattern of the RNA polymerase subunits from a blue dextran-Sepharose column. After addition of the enzyme to the column (see Methods), the column was washed with buffer F and then, after the $A_{280}$ (○) had fallen to less than 0.05, buffer F containing 0.6 M KCl was added. Fractions 1 through 30 contained 2.5 mL. All subsequent fractions contained 2.0 mL. Zinc was determined by atomic absorption and is plotted as the molar ratio of zinc to the subunits contained in the fractions (▲). Prior to zinc analysis all fractions were dialyzed for 48 h as described in Methods. The first elution peak contained α and β'; the second peak contained β (see Figure 2).

gether with a similar observation regarding the holoenzyme, this suggests that one zinc atom is bound more tightly than the other.

*Inhibition of RNA Polymerase Activity by Phenanthrolines.* Inhibition of the enzyme by isomers of phenanthroline was found to be similar to that reported for *E. coli* RNA polymerase (Scrutton et al., 1971). As shown in Table II, the presence of 1 mM 1,10-phenanthroline caused 40% inhibition, while the nonchelating isomer 1,7-phenanthroline was much less effective. Dialysis of the enzyme against 1 mM 1,10-phenanthroline for 1 day at 4 °C did not result in the loss of a significant fraction of the zinc.

TABLE II: The Effect of Isomers of Phenanthroline on RNA Polymerase Activity.

| Isomer | Concn (mM) | Act. as % of control |
|---|---|---|
| 1,10-Phenanthroline | 0.0 | 100 |
| | 0.1 | 103 |
| | 0.5 | 89 |
| | 1.0 | 60 |
| | 1.5 | 38 |
| | 2.0 | 30 |
| | 4.0 | 4 |
| 1,7-Phenanthroline | 0.0 | 100 |
| | 0.1 | 116 |
| | 0.5 | 109 |
| | 1.0 | 83 |
| | 1.5 | 77 |
| | 2.0 | 88 |

## Discussion

Several lines of evidence lead to the hypothesis that, for *E. coli* RNA polymerase, the β subunit contains the site(s) at which phosphodiester bond formation is catalyzed. Some mutant enzymes containing altered β subunits (Rabussay and Zillig, 1969) are not inhibited by the rifamycins, antibiotics which inhibit the initiation of RNA synthesis but which do not interfere with the formation of the enzyme–DNA complex (Hinkle et al., 1972). A chemically reactive rifamycin derivative attacks only the β subunit and the σ factor when added to *E. coli* RNA polymerase (Stender et al., 1975). Rifamycin has been shown to compete with GTP for binding to the low-affinity "initiation site" on the core enzyme (Wu and Goldthwait, 1969), suggesting that this site is located on the β subunit. The chelating agent 1,10-phenanthroline also appears to compete with GTP, and it has been proposed that the initiation site may contain tightly bound zinc (Scrutton et al., 1971).

The *B. subtilis* enzyme is very similar to the *E. coli* enzyme in subunit composition, zinc content, effect of stimulatory factors, and inhibition by the rifamycins and the phenanthrolines (Chamberlin, 1974). Rifamycin-resistant mutants of *B. subtilis* RNA polymerase have also been reported to contain altered β subunits (Linn et al., 1975). However, it has not been established that the *B. subtilis* β subunit has the same function as the *E. coli* β subunit. For example, by charge density electrophoresis, the *B. subtilis* β subunit migrates in a similar fashion to the *E. coli* β' subunit (Zillig et al., 1976).

The observation that the *B. subtilis* RNA polymerase contains two zinc ions which are firmly bound to the β subunit provides a rationale for understanding the binding of 1,10-phenanthroline (and possibly the rifamycins) to this enzyme. Two aromatic hydroxyl groups, as well as the two essential aliphatic hydroxyl groups, of the rifamycins are appropriately positioned to function as metal ligands (Riva and Silvestri, 1972).

The fact that, even in 6.5 M urea, the β subunit binds to blue dextran-Sepharose is consistent with the expectation that tightly bound zinc ions should stabilize the tertiary structure of the polypeptide and maintain the structural integrity of the nucleotide binding site (Vallee and Wacker, 1970).

Although zinc is associated with a large number of enzymes involved in nucleotidyl transfer, its function remains unknown. The observed frequency of occurrence suggests that zinc participates directly in catalysis, such as by acting as a Lewis acid during substitution at the 3'-hydroxyl group of the growing nuclei acid chain (Springgate et al., 1973). However, the metal might also alter the properties of amino acid side chains in the active region, or stabilize the structure of the enzyme–nucleic acid complex.

It appears that one of the zinc ions is bound more tightly than the other since prolonged dialysis against EDTA can remove 50% of the zinc from the *B. subtilis* holoenzyme, core enzyme, or β subunit. Incubation of the enzyme for several days in 7 M urea results in complete release of the zinc (Halling and Doi, unpublished observation); this may be a cause of low recovery of activity in previous reconstitution studies (Yarbrough and Hurwitz, 1974; Saitoh and Ishihama, 1976). The fact that zinc can be released from the β subunit should be considered in future dissociation–reassociation studies with the RNA polymerase subunits. The rapid method described above for obtaining β sununit (with its full zinc content and the property of binding to blue dextran intact) is a very attractive preparation procedure for reconstitution studies.

## Note Added in Proof

Recently completed reconstitution studies with RNA polymerase subunits from *B. subtilis* wild type and a rifampicin-resistant mutant have indicated that the larger subunit designated as $\beta'$ in this study determines the rifampicin-resistant phenotype. Therefore this larger subunit should be called the $\beta$ subunit and the zinc-containing subunit should be called the $\beta'$ subunit in order to be consistent with the prior nomenclature given to the *E. coli* RNA polymerase subunits.

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